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DIAMINE OXIDASE AND THE BIOGENESIS OF QUINOLIZIDINE ALKALOIDS IN THE YELLOW LUPINE

(*LUPINUS LUTEUS L.*)

BY

HUGH A. WHITE

©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Diamine Oxidase and the Biogenesis of Quinolizidine Alkaloids in the Yellow Lupine (Lupinus luteus L.)" submitted by Hugh A. White in partial fulfilment of the requirements for the degree of Master of Science in Plant Biochemistry.

ABSTRACT

It has been proposed that the action of diamine oxidase [diamine-oxygen oxidoreductase E.C. 1.4.3.6] is an important step in the formation of quinolizidine alkaloids. However, few investigations of lupine diamine oxidase have been carried out to study its possible role in quinolizidine biosynthesis. Diamine oxidase was isolated from seedlings of *Pisum sativum* and *Lupinus luteus* var. *Romulus* and partially purified. Assay techniques were modified for use with plant diamine oxidase and some of the properties of the partially purified enzyme were studied. It was shown that pea and lupine diamine oxidase were inhibited by substances known to inhibit diamine oxidases and were relatively unaffected by those known not to inhibit diamine oxidase. Both pea and lupine diamine oxidase were shown to have the ability to slowly oxidize L- and D-lysine.

A method was developed for the extraction of diamine oxidase from plant tissues. The changes in the levels of diamine oxidase activity in plants of *Lupinus luteus Romulus*, *Lupinus luteus Popularny*, and *Pisum sativum* from germination to an age of fourteen days were studied.

A thin layer chromatographic technique was developed for the separation of quinolizidine alkaloids. This method was used to check for the presence of alkaloids in the seeds of plants of the family *Leguminosae*, some of which were known to

form alkaloids, others of which were apparently alkaloid free. Seedlings of these plants were examined for diamine oxidase activity. Diamine oxidase activity was found both in plants known to accumulate alkaloids and in some that were not found to accumulate alkaloids in their seeds.

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A NOTE ON CONVENTIONS USED IN STRUCTURAL FORMULAE

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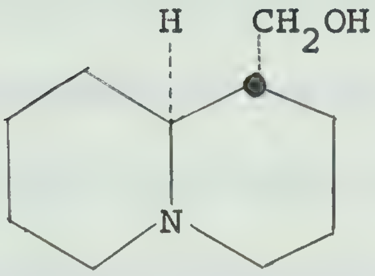
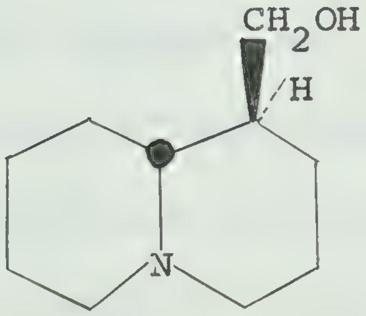




I. INTRODUCTION

A. THE PROBLEM AND THE INTRODUCTION TO IT

(1). Statement of the Research Problem

Research was undertaken to determine if diamine oxidase [diamine-oxygen oxidoreductase E.C. 1.4.3.6 (deaminating)] was involved in the conversion of lysine to quinolizidine alkaloids in plants of *Lupinus luteus*. Tracer studies have established that the quinolizidine alkaloids are formed from lysine and that the lysine is converted to a symmetrical intermediate before its incorporation into the alkaloid skeleton. This thesis concerns the next step in the elucidation of the biogenesis of the quinolizidine alkaloids, namely an examination of the enzymes involved in the conversion of lysine to these alkaloids.

It has been proposed that diamine oxidase plays an important part in this conversion. One hypothesis is that lysine is decarboxylated to 1,5-diaminopentane (cadaverine), which then undergoes an oxidative deamination catalyzed by diamine oxidase, to give aminoaldehydes. These oxidation products undergo spontaneous cyclization to form the heterocyclic precursors of the quinolizidine alkaloids. However, lysine decarboxylase activity and cadaverine have been found to be absent in plants known to synthesize quinolizidine alkaloids. *In vitro*, diamine oxidase is able to oxidize lysine directly.

It is thus possible that lysine itself is oxidized by diamine oxidase to form the heterocyclic alkaloid precursors in the plant. The major problem studied was to determine whether diamine oxidase was actually involved in the formation of these alkaloids and if it was, whether lysine, or its decarboxylation product, was the substrate for the enzyme. The isolation, partial purification, and study of certain of the properties of diamine oxidase was a first step towards a solution to this problem. Further, various plants known to form quinolizidine alkaloids were examined for diamine oxidase activity, and tracer and inhibitor studies were conducted to examine the role of the enzyme in the plant.

(2). The Introduction

The introduction to this thesis consists of six parts: a general introduction to alkaloids, a discussion of the quinolizidine alkaloids, a section on the plants used in this study, a review of the biogenesis of the quinolizidine alkaloids, relevant information on diamines, an extensive review of diamine oxidase, and finally a brief discussion of the possibility of transaminases, rather than diamine oxidase, having a role in the conversion of lysine to these alkaloids. The material in these sections is necessary to a good understanding of diamine oxidase in relation to the formation and possible functions of the quinolizidine alkaloids.

There is no reference available that summarizes the quinolizidine alkaloid information necessary to the biochemist. It is also my hope that this introduction will provide both a useful review and a key to the literature on these alkaloids and their formation for persons pursuing these studies further in the future.

B. GENERAL INTRODUCTION TO ALKALOIDS

(1). History

Since the earliest times certain plants have been used in human cultures to obtain specific effects on mammalian physiologies. Primitive (preneolithic) peoples still existing today possess extensive pharmacopeias of useful plants. Some plants produced poisons used in hunting and warfare, others produced curative or euphoric effects, and some others were valued for use in religious ceremonies for their unusual effects on human consciousness. A large proportion of the active principles in these plants has been found to consist of the materials now known to be alkaloids.

The first positive records available of the medical use of identifiable alkaloid-containing plants have been found on cuniform tablets in the library of King Assur-banipal (Assyrian: 668-626 B.C.) (1). The information on these tablets was probably recopied from much earlier records, possibly dating back to the early Sumerians (*floreat* 4,500-1,750 B.C.). The *Ebers Papyrus* (*c* 1,552 B.C.) contains recipes for the use of Egyptian medicinal plants while an inscription dating to the reign of Khufu (*c* 2,600 B.C.) may also contain references to alkaloid-forming plants with medicinal uses (2). (This reference (2) dates the reign of Khufu to 3,700 B.C. This date is incorrect. The reign of Khufu was *c* 2,600 B.C. (3).)

Literature pertaining to, and knowledge of, medicinal plants proliferated since the times of these first records: during the Greek and Hellenistic periods and into the middle ages herbals and "pharmacopeias" became increasingly common. However, no alkaloid was obtained in pure form until Sertürner crystallized morphine from opium in 1817 A.D. (4). In 1889 coniine (from hemlock) became the first alkaloid to have its structure confirmed by chemical synthesis (5). The determination of the structures and the syntheses of many alkaloids was intimately tied up with the development of classical organic chemistry. Today there is a vast part of the chemical literature devoted to studies of alkaloids, their chemistry, effects, and origins.

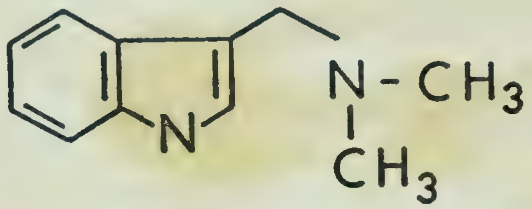
The development and prospects of alkaloid chemistry has been reviewed by Battersby (6). Henry's *The Plant Alkaloids* (18) contains useful and brief summaries on the chemistry and pharmacology of many of the major groups of alkaloids, but is unfortunately rather out of date, having been published in 1949. Perhaps the most useful and complete source of information about most aspects of alkaloid chemistry and pharmacology is the eight volume work, *The Alkaloids*, edited first by R.H.F. Manske and H.L. Holmes and now by Manske alone.

(2). Definitions of Alkaloids

The problem of exactly what constitutes an alkaloid has been discussed at some length by D.M. Graham (7) and by Mothes and Romeike (23). Most definitions seem to agree that an alkaloid must possess nitrogen (generally basic) and must occur naturally in plants (8). The word itself was coined by Meissner in 1819 (9). The best definitions are those based upon chemical properties and biosynthetic origin. A relatively simple definition would be that an alkaloid is a naturally-occuring compound that contains nitrogen (preferably in a heterocyclic linkage) and carbocyclic and/or heterocyclic structures, and that can be shown to be biologically derived (at least in part) from an amino acid.

A more complete series of definitions has been suggested by Hegnauer (10). He proposes three groups: true alkaloids, protoalkaloids and pseudoalkaloids. True alkaloids are defined as being N-heterocycles derived from a biogenetic amine formed through decarboxylation of an amino acid (e.g. lysine gives rise to quinolizidine alkaloids possibly through cadaverine, tyrosine leads to indolic alkaloids, and phenylalanine originates the isoquinoline group of alkaloids). Protoalkaloids are alkaloid-like compounds (nitrogenous and basic) that are derived from biogenetic amines, but which contain no heterocyclic system unless this is derived from

tryptophan. In this class fall the biogenetic amines themselves and their derivatives: gramine, which is an amine derived from tryptophan, and the betaines. Pseudoalkaloids constitute a



group of compounds that have all the properties of alkaloids but that have apparently no biosynthetic relation to amino acids. This class contains compounds whose carbon skeletons are derived from monoterpenes, diterpenes, sterols, and acetogenins. The lycopodium alkaloids originate from acetate units and are thus acetogenins (or polyketides) rather than true alkaloids (11). The major portions of the skeletons of the Solanum and Veratrum alkaloids are steroid in nature (12) so these alkaloids are better classified as pseudoalkaloids. Very large differences exist among the origins and probably the functions of the true alkaloids and pseudoalkaloids. The biosynthetic origin of a compound must be known, then, before it can be properly classified. In common use the term alkaloid still relates to any basic, nitrogen-containing compound of plant origin that has not been assigned some metabolic function. In this thesis the term alkaloid will be used to refer to a

compound found naturally in plants that contains nitrogen in a heterocyclic skeleton and that is formed biologically from an amino acid precursor.

(3). Occurrence

Compilations of alkaloid bearing orders and families of plants are to be found in references 7, 10, 13, 15, and 23. True, or at least pseudoalkaloids, are to be found in all of the plant phyla (with the possible exception of the algae), but true alkaloids are comparatively rare in the lower plants (10). The fungi yield several classes of alkaloids. Alkaloids have been isolated from *Amanita*, *Aspergillus*, *Claviceps* (ergot alkaloids), *Fusarium*, and *Penicillium* (14). Alkaloids have also been reported to be present in a lichen (14) but which of the symbionts is responsible has not been determined. Steroidal alkaloids have been found to be produced by some types of frogs (16).

Among the higher plants the distribution of alkaloids is uneven - many families are alkaloid-poor while others contain many alkaloid-forming genuses (10). The families *Centrospermae*, *Magnoliales*, *Ranunculales*, *Papaveraceae*, *Leguminosae-Papilionatae*, *Rutaceae*, *Bunaceae*, and *Gentianales* are particularly richly endowed with alkaloid-synthesizing members (10). Other families contain scattered alkaloid-forming genuses. The search for alkaloids has just really

begun and it seems likely that increasingly large numbers of families will be found to elaborate alkaloids when the newer, highly sensitive methods of detection and isolation are applied. It has been estimated that 10 to 20% of all the higher plants produce alkaloids (14). This figure is probably too low and the actual percentage is anyone's guess. It may eventually prove that nearly all plants produce alkaloids! Nicotine has been detected in tomatoes by improved methods (14) and quinolizidine alkaloids have been found in garden peas (17).

There seem to be no good correlations between habitat and occurrence of alkaloid-bearing plants. Every environment that supports vascular plants has a fair share of alkaloid-producers (13).

In the past few years the presence of "natural products" (terpenes, flavonoids, anthocyanins, alkaloids, etc.) has been used as an aid to the systematic classification of plants. This increasingly popular aid to classification is now known as chemotaxonomy and has frequently been reviewed (10, 19, 20, 21, 22). There are dangers in using the occurrence of alkaloids as a sole basis for classification. There are cases where the same compound occurs in two taxa that are not particularly closely related and the same biosynthetic pathway has been shown to be active (e.g. sparteine occurs both in

Lupinus and in *Chelidonium*). N-methylcytisine is known to occur in members of the *Berberidaceae*, *Leguminosae*, and *Scrophulariaceae* (10). Also, there are cases where clearly related groups of plants differ widely in their natural product composition. Anabasine is produced in the unrelated taxa *Chenopodiaceae* and *Solanaceae* and has been shown to be synthesized by different pathways in each family (10). Nicotine is also widely spread (occurs in at least five different families) (14). Often most valuable taxonomically are groups of related compounds differing only in substitution pattern (19) (e.g. closely related species of duckweed show large variations in their flavonoid content; the differently substituted quinolizidines are of taxonomic interest among the *Leguminosae*). The condition and environment of the plant, as well as its stage of development is of considerable importance in determining its alkaloid spectrum and content (14, 22). The compounds selected for chemotaxonomic study are of great importance to the success of the study. Only certain alkaloids are suited in this regard. Further knowledge of the biosynthetic pathways (including studies of the enzymes involved), of the physiology and metabolism of the alkaloids, and of the inheritance of the ability to form alkaloids is a necessity before chemotaxonomic studies can be made fully valid.

(4). Location in the Plant, Physiology, and Biogenesis

Alkaloids may be accumulated in virtually any part of the plant. Alkaloids may be found in seeds, leaves, stalks, flowers, fruits, roots, storage organs, and even in the bark (13, 14, 22, 23, 24, 25). They may constitute anything from the merest trace to some 10 or 12% by weight (berberine in barberry root bark (13, 24)). A careful distinction must be made between the site of synthesis and the site of accumulation (22, 25) - these may be very different! The best demonstration that a particular organ is a site of alkaloid formation, rather than accumulation, is the synthesis of the alkaloid in a culture of that organ (14, 22, 25, 28). Grafting experiments have also been used in locating the organs of alkaloid formation and accumulation (14, 22, 23, 25). There are no general rules to be drawn regarding the locales of alkaloid synthesis. In some families only the roots are active in alkaloid production, while in others the shoots and leaves exclusively are active (14). The exact site of formation has often been a subject for bitter debate (22) and in many cases has not yet been resolved.

Histochemical studies with the use of classical alkaloid precipitants (e.g. iodine-potassium iodide, heavy metal salts, picric acid, etc.) to show the location of alkaloids in plant tissues have been common (23, 24).

Changes in alkaloid concentration in developing tissues have been studied (24). Where alkaloids are present their concentrations are found to be high in growing or metabolically active tissues, in epidermal tissues, in vascular sheaths and associated tissues and in latex vessels, where present (24). Alkaloids seem invariably to occur within the vacuole of the cell (24).

Generally speaking, the conclusions obtained with these classical methods may not be entirely valid. It is difficult, if not impossible, to fix and prepare tissue for sectioning and staining by the traditional methods without at least partial loss or movement of the water-soluble alkaloids. Studies on living tissues necessarily require thick sections or extensively damaged tissues with resultant loss of structure and resolution. Furthermore, there have been few studies on the usefulness of precipitating agents for the detection of trace quantities of alkaloids. Classical methods show clearly the presence of accumulated alkaloids in the vacuole, but are probably insufficient to show the presence or absence of small amounts of alkaloid in the cytoplasm. The solubility product of the alkaloid precipitate would have to be exceedingly small for a detectable precipitate to form at the alkaloid levels that are likely to exist in the cytoplasm. A histochemical method has been developed that utilizes rapidly

frozen and freeze-dried tissue, thus preventing alkaloid loss or movement during processing (26). The resolution so far obtained with this method is not sufficient to accurately localize the alkaloid within the cell. It appears, however, that some alkaloid may be present in the cytoplasm. The only electron microscope study in alkaloid histochemistry reports on the presence of quinolizidine alkaloids in Baptisia tissues (27). The method employed glutaraldehyde fixation followed by a powerfully disruptive alkaloid precipitant. The results so obtained may not be entirely a true representation because of solubility losses during processing and the strong staining reagent used. Alkaloidal precipitates were found to be present in the vacuole as well as small deposits in the cytoplasm. Much more research should be done to localize the exact sites of alkaloid formation and storage in the cell.

Very little work has been done on the preparation of enzyme or subcellular systems active in alkaloid biosynthesis or metabolism. Some studies concerning gramine biosynthesis by soluble enzyme systems and subcellular fractions have been carried out (29). A soluble enzyme fraction from barley was found to be most active in gramine biosynthesis. Gramine, however, is not a true alkaloid - its N-heterocyclic skeleton is directly derived from tryptophan.

Alkaloids are not without effect on the physiology of plant tissues, although their effects on animal tissues have been studied much more thoroughly. The effect of colchicine on plant growth and cell division is well known (30). Grafting experiments have shown that excessive nicotine causes necrosis, chlorosis, and stunting when roots of a plant that forms large amounts of nicotine (e.g. *Nicotiana spp.*) are grafted to shoots of plants low in nicotine (e.g. tomato or belladona). These experiments show that tomato and belladona differ from *Nicotiana* in their ability to *tolerate* nicotine, not to form it (14). The resistance of plants to alkaloids may be of taxonomic importance. It is possible that resistance to an alkaloid may indicate a potential (even if genetically blocked, say by the mutation of a gene for an enzyme necessary to the biosynthesis) for the formation of alkaloids of that class. Studies on the effects of different classes of alkaloids on plant tissues have been largely neglected, but promise to be of great interest from the taxonomic point of view as well as being useful in studies of the functions and metabolism of the various classes of alkaloids. One wonders how plants of the autumn crocus can carry out normal cell division in the presence of colchicine! Is a strict compartmentation mechanism in effect, or is the

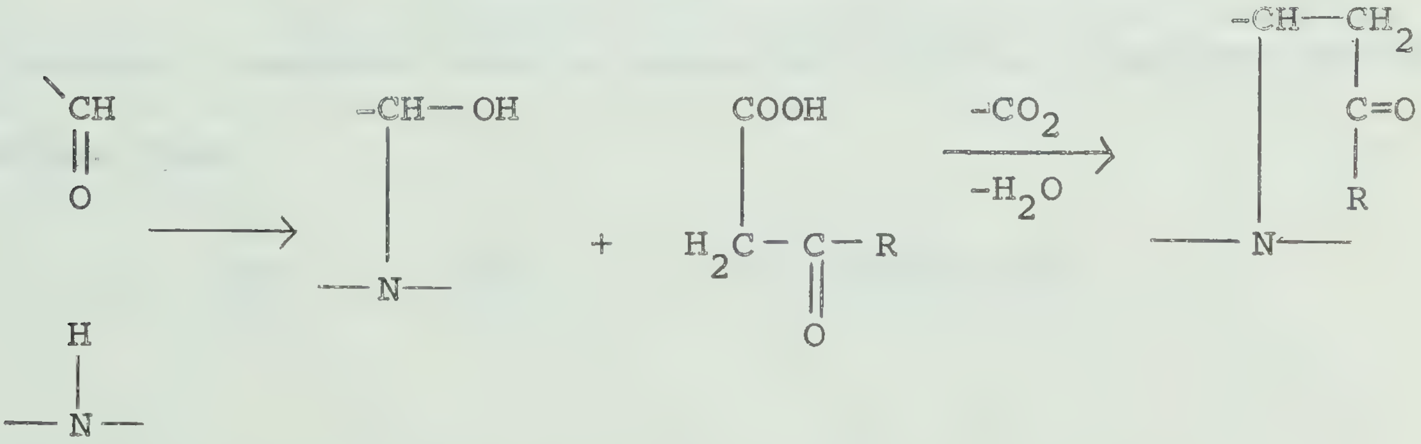
action of the colchicine neutralized by some sort of control system? Similar questions apply to all alkaloid-forming plants.

The preparation of reviews dealing with alkaloid biosynthesis has recently become a popular pass-time. The following references contain general reviews of alkaloid biosynthesis (underlined reviews are the most useful): 8, 14, 21, 22, 23, 25, 32, 33, 34, 35, 36, 37, 38, 39, 40 and 41. This is only a partial listing - reviews of the biosynthesis of particular alkaloids have been excluded and only those that are recent and/or important, and readily available in English or German, have been listed. Ramstad and Agurell (31) point out that the terms 'biosynthesis' and 'biogenesis' are not interchangeable. Biogenesis indicates the formation of a compound as it occurs naturally in cells; biosynthesis refers to the active formation (also by living tissues) of a compound by outside design or manipulation. As most of the biogenetic schemes proposed and studied have been obtained by forcing plants to biosynthesize compounds from known precursors, it is extrmely difficult to apply this semantic nicety. In the majority of the published literature, and and in this thesis, the terms are used interchangeably.

It appears from recent work that only a limited number of chemical reactions are involved in the formation of

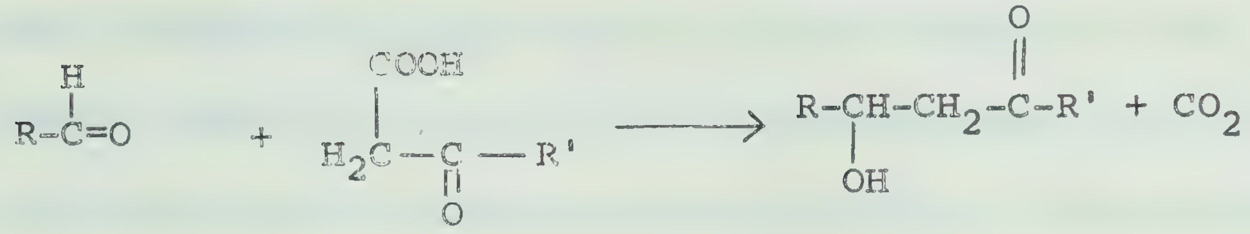
the N-heterocyclic skeletons of alkaloids. Leete (32) lists the reactions involved as being: Schiff base formation in the complete synthesis of alkaloids (i.e. formation of the heterocyclic skeleton and reactions following this), the Mannich reaction, various oxidative reactions (e.g. oxidative coupling, dehydrogenation, epoxide formation, etc.), reductions, aldol condensations, decarboxylations, dehydrations, substitutions, and rearrangements. He gives known examples of all of these reactions in alkaloid formation (32). The majority of these reactions are concerned with steps preliminary or subsequent to the actual, crucial formation of the alkaloid skeleton. Mothes and Schütte (34) summarize the basic reactions in the formation of the alkaloid skeletons as being:

(1) Formation of "aldehyde-ammonias" from an aldehyde carbonyl and the hydrogen of a primary or secondary amine. This produces a carbinolamine that condenses with active methylene groups of ketones or of β-oxo acids. e.g.



An imine, -CH=N-, can be substituted for the aldehyde in these reactions.

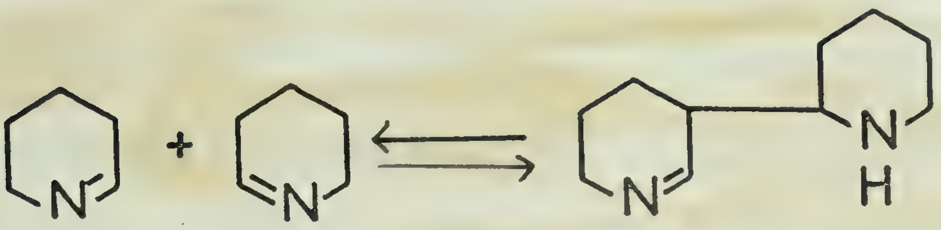
(2) Aldol condensations between aldehydes and β-oxo acids. e.g.



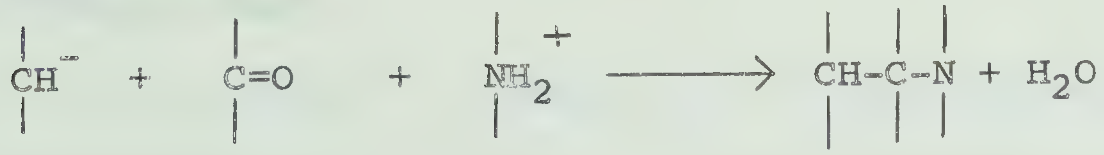
$$\mathrm{R{-}\overset{H}{\overset{|}{C}}{=}O} + \mathrm{H_2\overset{COOH}{\overset{|}{C}}{-}\underset{O}{\underset{\|}{C}}{-}R'} \longrightarrow \mathrm{R{-}\underset{OH}{\underset{|}{CH}}{-}CH_2{-}\overset{O}{\overset{\|}{C}}{-}R'} + CO_2$$

(This reaction could serve to form the heterocyclic ring if the aldehyde and active methylene group were at the ends of the same molecule. Nitrogen presumably would be present in the molecule being cyclized).

(3) Condensations of an aldol type between -CH=N- and the reactive methylene group of Δ'-piperideine. e.g.



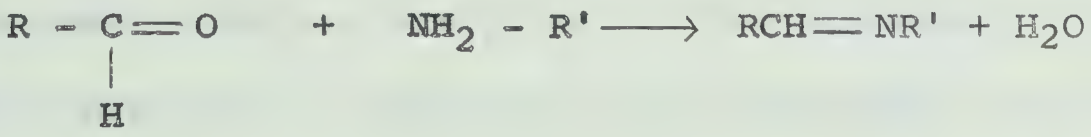
(4) A very common cyclization is the reaction of a carbonyl compound with an amine and with an 'electronegative' carbon atom.



$$\underset{|}{\overset{|}{\mathrm{CH}}}{}^{-} + \underset{|}{\overset{|}{\mathrm{C}}}{=}\mathrm{O} + \underset{|}{\overset{|}{\mathrm{NH_2}}}{}^{+} \longrightarrow \underset{|}{\overset{|}{\mathrm{CH}}}{-}\underset{|}{\overset{|}{\mathrm{C}}}{-}\underset{|}{\overset{|}{\mathrm{N}}} + \mathrm{H_2O}$$

This is the Mannich reaction and is discussed extensively by Leete (32). The chemistry of the Mannich reaction has been reviewed by Blicke (42). A good example of the Mannich reaction occurs in the proposed scheme for the biogenesis of the quinolizidine alkaloids. A dialdehyde amine produced from cadaverine or lysine undergoes condensation to give the parent quinolizine alkaloid, lupinine (32).

Mannich reactions have also been proposed to be important in the formation of the pyrrolizidine, indole, and tropane alkaloids (32, 34). The first step in the Mannich reactions is Schiff base formation which itself is important in the formation of the Erythrina and Amaryllidaceae alkaloids (32). The condensation of an aldehyde with an imino group produces a 'Schiff base'.



$$R - \underset{\displaystyle H}{\underset{|}{C}} = O \quad + \quad NH_2 - R' \longrightarrow RCH = NR' + H_2O$$

This reaction converts a carbonyl double bond to a C=N bond, while the completed Mannich reaction forms two single bonds (32, 42).

Various other coupling reactions have been studied in regard to alkaloid biosynthesis. Leete's review (32) gives a good summation of the oxidative and reductive (aldol condensations are included) reactions probably involved in alkaloid formation. Oxidative coupling reactions are also reviewed in references 33, 34, 35 and 43. As these reactions are not important in quinolizidine alkaloid biogenesis they will not be reviewed here.

Following the initial formation of the alkaloid skeleton, many other reactions may occur, such as dehydrogenations, dehydrations, decarboxylations, various hydroxylations and oxygenations, reductions, esterifications, methylations and demethylations, and even peptide bond formation, which give rise to the large numbers of differently substituted members of the same basic family. It has been proposed that methylation and acetylation occur before ring closure, thus blocking possible alternate reactions, but this has not yet been shown to be a general case (35). Often these secondary reactions are species specific (e.g. some species form cytisine alone, while others form both N-methyl cytisine and cytisine, while yet others accumulate only N-methyl cytisine). Alkaloids once formed may also undergo metabolic interconversions as well as degradations to non-alkaloid materials (24, 44, 45).

It cannot be overemphasized that most theories of alkaloid biogenesis are based upon evidence that is primarily chemical, rather than biochemical (7, 32, 34). Most of the reactions discussed have been carried out *in vitro* under conditions intended to resemble those in the cell (*e.g.* high dilution, aqueous solutions, near-neutral pH). The existence of a working model reaction for a proposed biosynthetic scheme in no way proves that the scheme actually functions in the plant - it only shows that such a reaction could possibly occur. Almost all of the chemical reactions carried out in the cell are enzyme catalyzed, rather than being spontaneous. It is also very likely true that at least some of the reactions involved in alkaloid formation are enzyme catalyzed. In general, alkaloids have several centers of optical activity and often have, therefore, large numbers of optical and conformational isomers. It is very often found that a particular plant accumulates only one enantiomorph of a pair of stereoisomers (e.g. *Lupinus sericeus* Pursh. synthesizes only *d*-α-*iso*lupanine, *d*-sparteine is formed in *Genista monosperma* Lam. while *l*-sparteine is found in *Lupinus mutabilis* Sweet and *dl*-sparteine is found in *Adenocarpus hispanicus* D.C. (46). There are also cases where a racemic alkaloid is accumulated and other cases where both enantiomorphs are present, but one is present in a larger

amount than the other (e.g. see tables in references 46, 47 and reference 64. Most chemotaxonomic work with alkaloids has been done simply on the basis of the presence of a certain alkaloid. Which stereoisomeric form is present has rarely been determined and is probably of equal importance to the simple presence of a particular alkaloid. The chemical reactions postulated and tested *in vitro* almost always give completely racemic products (32, 34, 42). The optical activity of the *in vivo* products (of course, assuming the reaction *in vivo* is similar to that *in vitro*) is taken as evidence of the enzyme mediation of the reactions *in vivo* (32, 34) as most enzyme-catalyzed reactions are highly stereoselective. It is possible that reactions similar to those proposed from model reactions are either enzyme catalyzed or coupled to enzyme reactions. For example, if *d*-A is an alkaloid accumulated by a plant and that is formed from an optically inactive precursor P through the optically active intermediates Q and R (R contains the completed alkaloid skeleton and differs from A only in substitution), it is possible that *d*-A could result either from a series of stereospecific enzyme reactions, or from a (possibly non-enzymic) reaction giving racemic Q (or racemic Q and R), which then are acted upon by stereospecific enzymes. The unreacted intermediate isomer might then be recycled back

to the starting material or could be metabolized in some fashion to other, non-alkaloid, materials. It is also possible that $\underline{dl}$-A could be formed, but the $\underline{l}$-isomer is metabolized leaving an accumulation of $\underline{d}$-A. These possibilities are shown in the equations following. A stereospecific reaction is indicated by 's', 'ns' indicates a non-stereospecific reaction.

$$\mathrm{P} \xrightleftharpoons{\mathrm{s}} \underline{\mathrm{d}}\text{-Q} \xrightleftharpoons{\mathrm{s\ or\ ns}} \underline{\mathrm{d}}\text{-R} \xrightleftharpoons{\mathrm{s\ or\ ns}} \underline{\mathrm{d}}\text{-A}$$

$$\mathrm{P} \xrightleftharpoons{\mathrm{ns}} \underline{\mathrm{dl}}\text{-Q} \xrightleftharpoons{\mathrm{s}} \underline{\mathrm{d}}\text{-R} \xrightleftharpoons{\mathrm{s\ or\ ns}} \underline{\mathrm{d}}\text{-A}$$

$$\mathrm{P} \xrightleftharpoons{\mathrm{ns}} \underline{\mathrm{dl}}\text{-Q} \xrightleftharpoons{\mathrm{ns}} \underline{\mathrm{dl}}\text{-R} \xrightleftharpoons{\mathrm{s}} \underline{\mathrm{d}}\text{-A}$$

$$\begin{tikzcd}
\mathrm{P} \arrow[r, shift left, "\mathrm{ns}"] & \underline{\mathrm{dl}}\text{-Q} \arrow[l, shift left] \arrow[r, shift left, "\mathrm{ns}"] & \underline{\mathrm{dl}}\text{-R} \arrow[l, shift left] \arrow[r, shift left, "\mathrm{ns}"] & \underline{\mathrm{dl}}\text{-A} \arrow[l, shift left] \arrow[r, "\mathrm{s}"] \arrow[d] & \underline{\mathrm{l}}\text{-x} \\
 & & & \underline{\mathrm{d}}\text{-A}\ \text{accumulates} &
\end{tikzcd}$$

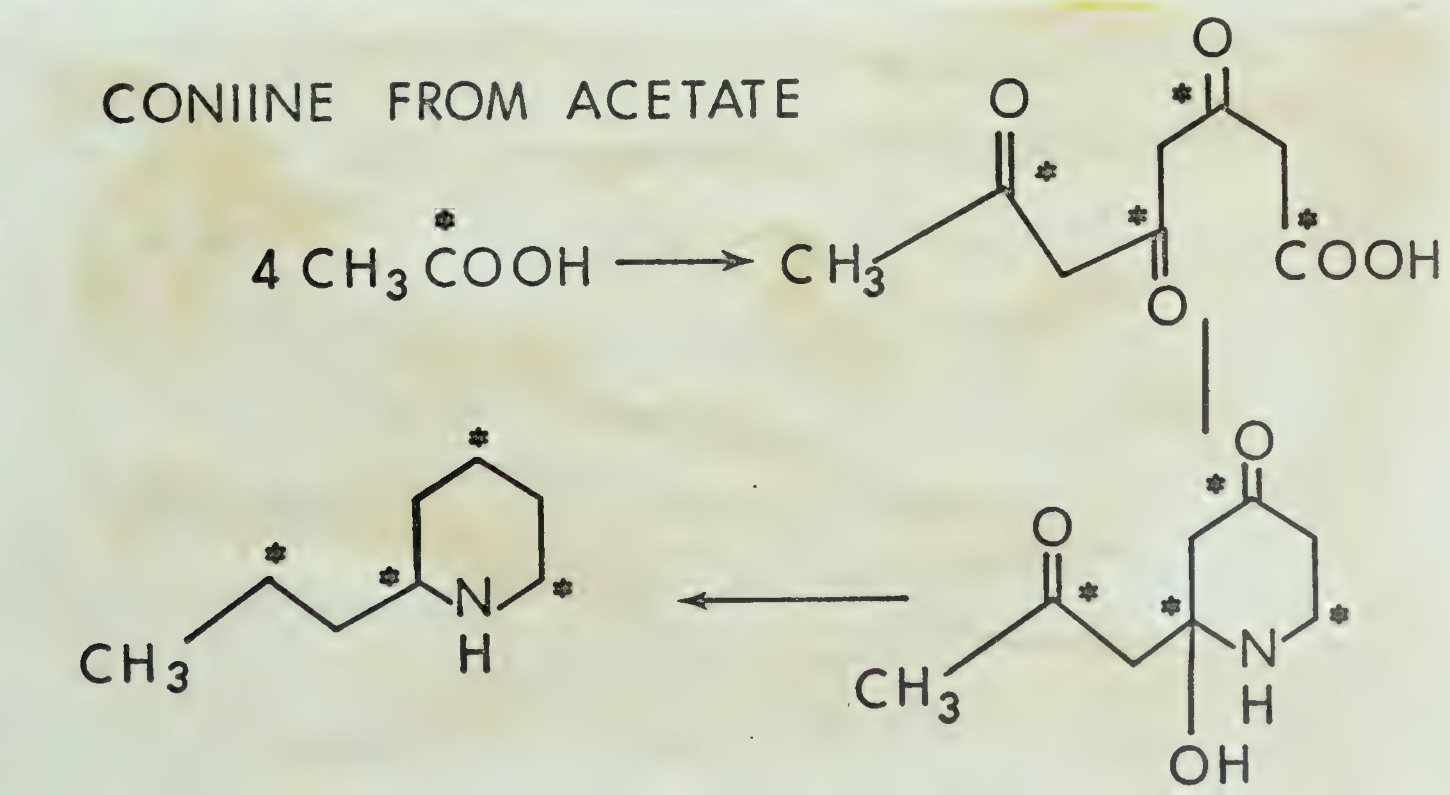
It has been conclusively shown that the formation of alkaloids is under genetic control (22, 48, 49). This is excellent evidence for the involvement of enzyme reactions in alkaloid biogenesis.

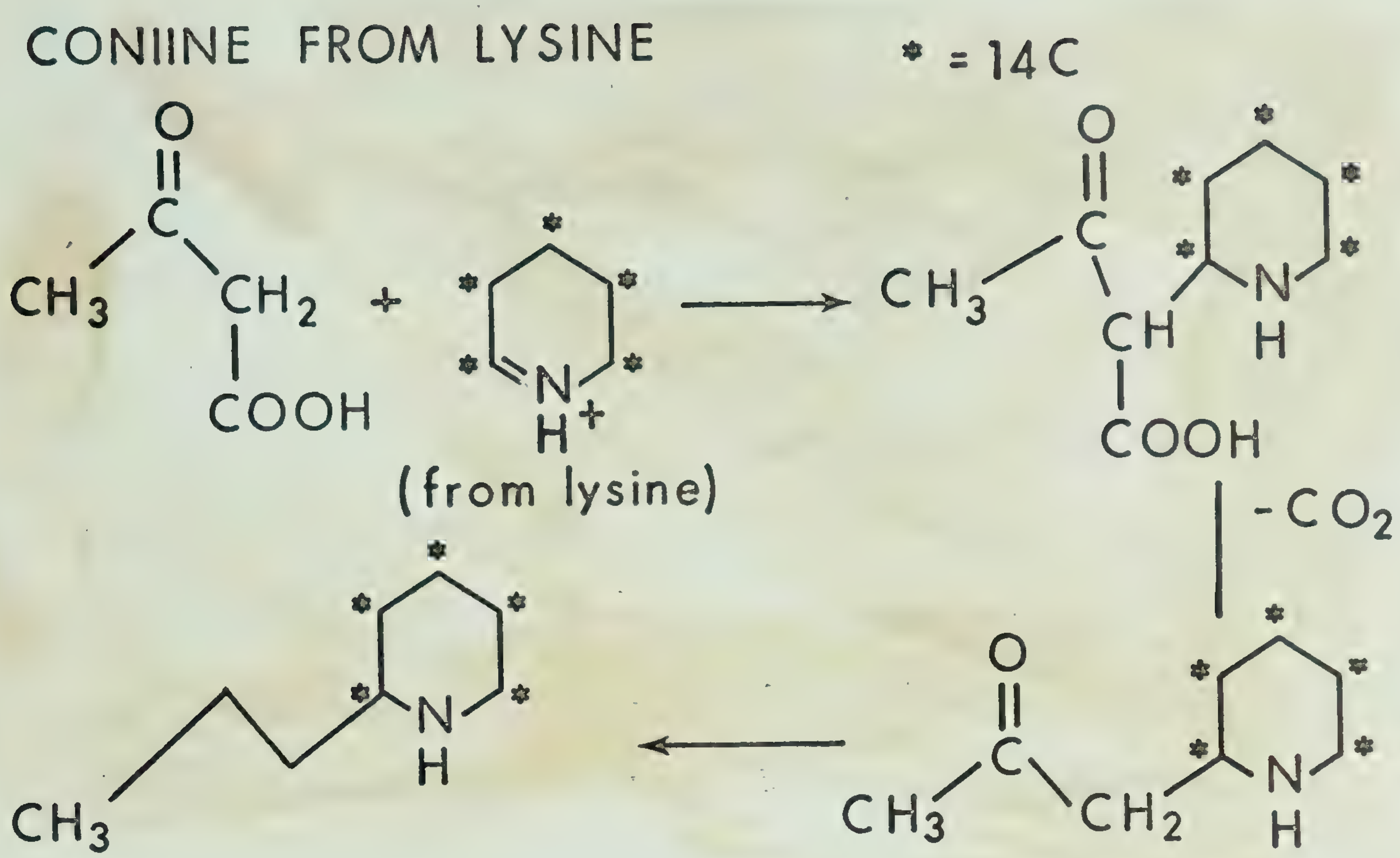
The general procedure in studies of alkaloid biosynthesis is to administer a specifically labelled precursor (usually ^{14}C, although ^{3}H and ^{15}N have been used) to the plant and, after an appropriate period, the alkaloids are isolated and the position of the label determined by

degradation. Feeding is most frequently accomplished by a fine thread drawn through the stem. The thread acts as a wick and virtually total uptake of the labelled compound can be accomplished by 'washing' with water after uptake of the precursor solution (36). This method avoids microbial contamination which has been a problem with many other feeding methods. Others use $^{14}CO_2$ in attempts to trace the whole pathway from carbon dioxide to the alkaloids (36). Feeding experiments are most often carried on for long periods of time (experiments measured in weeks are not too rare). These long periods are often necessitated by the relatively slow rate of alkaloid formation from the added precursors and by the losses involved in the isolation and degradative procedures. Such long term experiments constitute a danger, however, as it is possible for the precursor fed to be completely degraded and converted to other, possibly unrelated, materials which then go to form the alkaloid. In most cases this difficulty is minimized by the feeding of specifically (as opposed to uniformly or randomly) labelled precursors. The isolation of the alkaloid with radioactive carbon in the positions predicted or explained by the hypothesized pathway is fairly good evidence for a direct conversion to the alkaloid, rather than an indirect conversion via some other pathway.

In no case have the enzymes catalyzing the reactions of a proposed biogenetic pathway for an alkaloid been adequately studied. Only a very few workers have even attempted to look for the enzymes involved. The biosynthetic proposals made cannot be regarded as proved until the enzymes involved have been studied and demonstrated to act *in vivo* and *in vitro*.

The results and interpretations of labelling experiments are often open to question and debate. For example, lysine-U-^{14}C fed to hemlock plants gave labelled coniine and a scheme was proposed for its biosynthesis from Δ'-piperideine (derived from lysine) and acetoacetate (34). This scheme fitted well with knowledge of other pathways involving Δ^{1}-piperideine for other groups of alkaloids (*e.g.* quinolizidine and pyrrolizidine). Recent work has indicated that perhaps lysine is not such a good precursor and acetate has been proposed (36).





CONIINE FROM LYSINE

Should this prove to be the case, then coniine is probably not even a true alkaloid! The evidence available is not really sufficient to prove either scheme. Leete mentions that nicotiana plants can utilize 'unnatural' compounds to form alkaloids and that under some conditions 'aberrant' synthesis can occur when certain materials are fed (50). There are few, if any, of the proposed biogenetic pathways for alkaloids that can be regarded as unequivocably demonstrated.

(5). Possible Functions for Alkaloids

The reasons why plants synthesize alkaloids (and most other 'natural products') is a knotty problem. Proposed roles in the plant range from alkaloids being

functionless end-products of metabolism to proposals of hormone roles for them (7). It is impossible to generalize functions for alkaloids as there is such a wide assortment of different, unrelated compounds relegated to this class. Often one of the chief reasons a material is classed as an alkaloid is that it has no discernable function! Discussions of alkaloid function are to be found in references 7, 22, 23, 24, 25, 51, 52 and 58. It appears likely that different functions may be served by the different classes of alkaloids. It is also possible that the same alkaloid occurring in unrelated species may serve either dissimilar or similar ends.

Most of the older theories based upon some sort of protective function for alkaloids have been discarded, at least as general functions (51). Although it is quite possible that certain alkaloids may protect the plants forming them from some diseases or herbivores. Graham (7) has pointed out that only a small selective advantage need be conferred by an alkaloid-forming mutation for the character to eventually spread through a whole population by genetic drift. This argument neglects the fact that, in most cases, several enzymes are required to form an alkaloid from its amino acid precursor. Although some of these enzymes may already exist in the plant serving other functions, most of them are probably highly specific and function only

in alkaloid biogenesis. This being the case a considerable selective advantage (such as has not been demonstrated) would have to exist for the genes for several enzymes to evolve through mutation and spread through the population. For a single mutation to give rise to alkaloid formation seems unlikely. Although, if alkaloids of a certain group were formed in all plants and rapidly utilized, a single mutation could account for their accumulation in certain genuses. Some of the functions proposed for the quinolizidine alkaloids are discussed in a later section.

The idea that alkaloids are "flotsam thrown up on the metabolic beach" has little support (51). The chief argument against it is that alkaloid biogenesis is an energy-utilizing process involving often several steps. The continued existence of such an elaborate system to produce wastes seems very unlikely. Alkaloids are generally associated with growing or otherwise active tissues (24, 52). As alkaloids are often toxic (to some extent even to the tissues producing them when they are fed from outside) it does not seem reasonable that toxic wastes would be accumulated in growing cells even if the wastes were produced there. Alkaloids are even frequently translocated to the most actively growing parts of the plant (22). The work of Fairburn and his coworkers (as well as that of other groups) with hemlock,

opium poppies, and Atropa indicate that the alkaloids are in a very rapid state of flux. Measurable changes in alkaloid content occur over periods measured in a few hours and evidence has been given for rapid conversions among alkaloids, and of alkaloids to non-alkaloidal materials (44, 45, 53, 54). Complete and periodic disappearance of the major alkaloids has been observed (52). These studies certainly indicate active rather than passive roles for these particular alkaloids.

Recently evidence has been proposed that some fungal alkaloids apparently act as growth regulators in the fungi that produced them (55). One alkaloid, trigonelline (N-methyl pyridine-3-carboxylate) has been suggested to contain a high-energy bond (56). This suggestion is based on studies of the effect of this alkaloid in increasing the heat produced during seed germination (during which the alkaloid is demethylated). The authors state that their evidence does not prove the idea, but they feel that it is highly probable. The biogenesis of nicotine by Nicotiana and of ricinine by Ricinus communis is related to pyridine nucleotide formation and metabolism (62, 63). The exact interrelationship between these alkaloids and the pyridine nucleotides has not been fully elucidated, but it is clear that the pyridine nucleotides can be converted to the alkaloids.

The possibility that alkaloids may be far more widely spread than is now known was mentioned above. If a certain class of alkaloids should be shown to be very widely spread, then this too might argue for a function, possibly specific, for that class. Perhaps some classes of alkaloids serve different and specific functions in all plants, and only certain plants accumulate abnormally large (i.e. measurable by the commonly used methods) of these substances normally present only in traces.

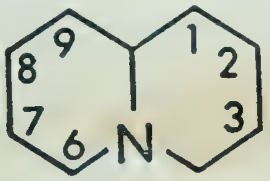
The fact that alkaloids contain nitrogen in heterocyclic linkage is of interest. In most cases the nitrogen has a free lone pair of electrons and potentially (frequently at physiological pH) bears a positive charge (i.e. when protonated). The charge, and more importantly, the pair of electrons (when unprotonated) can often be delocalized over the heterocyclic system (52). Such charge delocalization is a property common to many biologically active molecules (57). The often complex and rigid three-dimensional structures of many alkaloids may serve to fix the nitrogen into a position where it can interact with some other species. It has been shown that indole compounds ,which would include the many indole alkaloids, can act as electron donors in the formation of electron-donor-acceptor complexes and that, in such complexes, the relative

orientations of the molecules is of great importance (61) . The substituents (e.g. carbonyl, carboxyl, hydroxyl, and amino groups etc.) on the alkaloid skeleton could be of use in orienting the alkaloid molecule relative to perhaps an enzyme active site by electronic interactions to allow the heterocyclic nitrogen to fulfill some catalytic function. Alkaloids could also be bound by apolar interactions. Moreover, there do not seem to be any good reasons why alkaloids could not co-ordinate metal ions, either free or those bound into metalloporphyrins. There have been no recent investigations at all into possible roles of alkaloids as active cofactors in metabolism. It has been shown that alkaloids can interact with proteins (59) and that a pyridine polymer can interact with DNA (60) - this indicates a possibility that alkaloids (at least those with pyridine rings) could interact with nucleoproteins. Future research into alkaloid functions no doubt will bring many surprises.

C. QUINOLIZIDINE ALKALOIDS

(1). Definition, General Introduction, Structures

Quinolizidine alkaloids contain (actually or in modified form) the ring structure of the parent compound, quinolizidine (47):

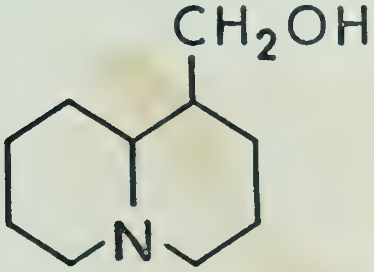


Numbering after 46

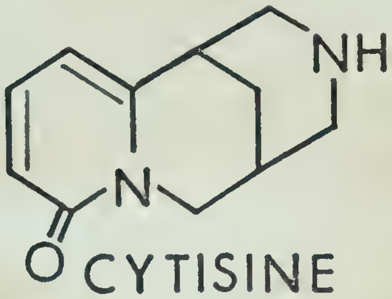
Quinolizidine is also known as 1-azabicyclo [4.4.0] decane, norlupinane, and octahydropyridocoline (47). The alkaloids containing this structure are alternately known as the lupine alkaloids or the Papilionaceous alkaloids. The term quinolizidine alkaloids is to be preferred as these alkaloids are not entirely restricted to the genus *Lupinus* or even to the subfamily *Papilionaceae* (47) of the Leguminosae family. The appellation, 'leguminous alkaloids' has also seen use, but other, non-quinolizidine alkaloids are found among the legumes (even in the genus *Lupinus*) (46, 47).

Several reviews on the quinolizidine alkaloids are available (18, 46, 47, 65, 66, 67, 82). Table I shows the structures of the most basic (and some interesting, but less common derivatives) of the quinolizidine alkaloids and the names of example species known to form them. The classification proposed by Crow (65) is a convenient one.

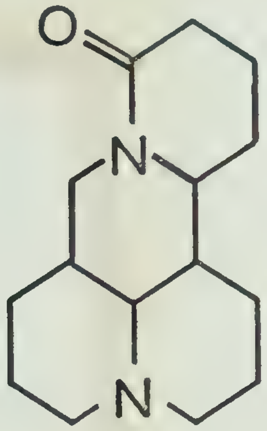
He divides the quinolizidine class into three groups according to the number of rings. The lupinine group has two rings, the cytisine group three, and the sparteine/matrine group four. The parent alkaloids are shown below.



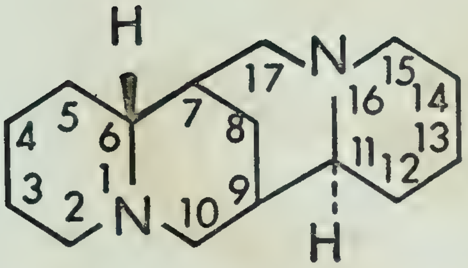
LUPININE



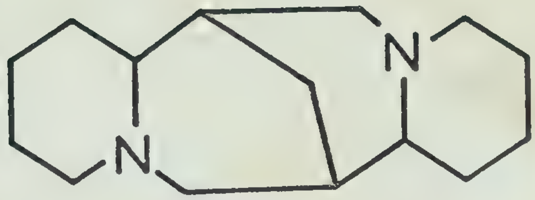
CYTISINE



MATRINE



SPARTEINE
(Numbering after 46, 65)



SPARTEINE alternate representation.

Table I is by no means complete: there are a large number of other quinolizidine alkaloids, but these are for the most part closely related to those shown in the table. New alkaloids in this class are still being discovered and studied. The name given a particular new alkaloid often changes as its similarities to known alkaloids are discovered. Several quinolizidine alkaloids are known only by numbers. Of course, each worker has used a different numbering system!

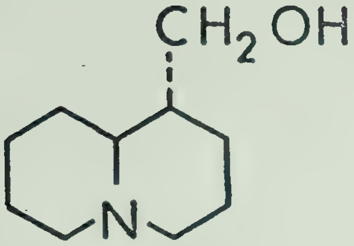
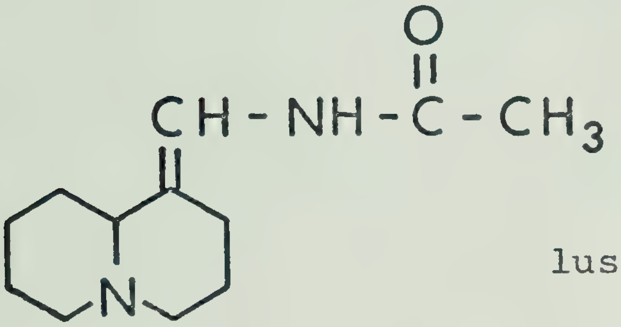
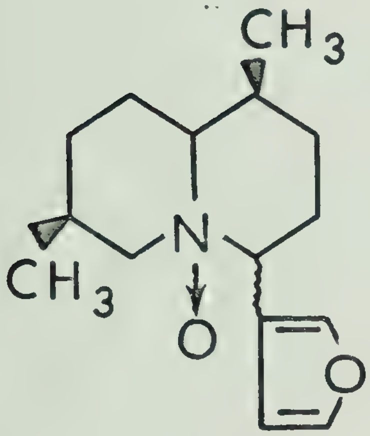
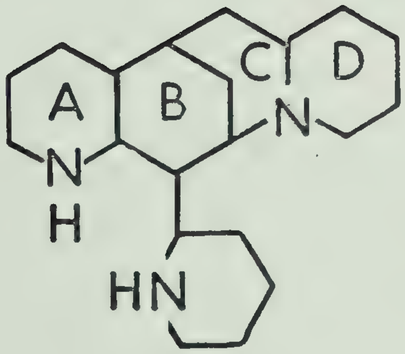
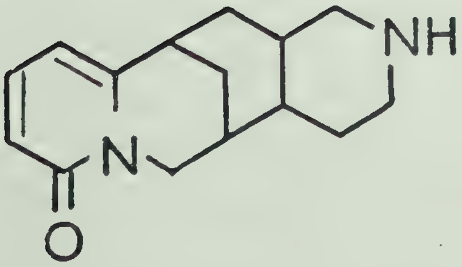
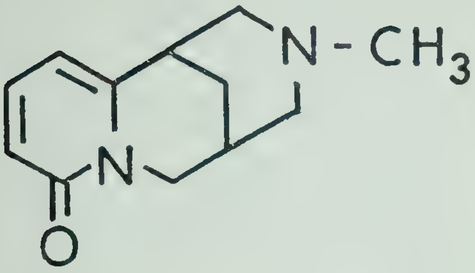
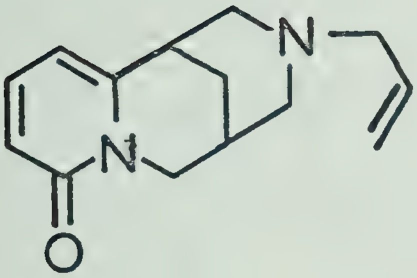
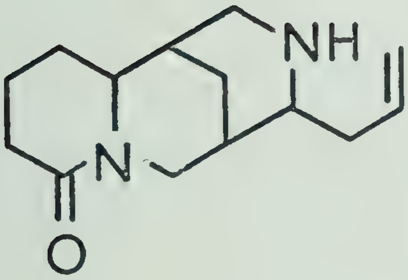
Structure	Name	Example plant	Reference
	LUPININE GROUP		
	lupinine	*Lupinus luteus* L.	47
	lusitanine	*Genista lusitanica*	68
	nupharidine	*Nuphar japonicum*	69, 74
	ormosanine	*Ormosia* sp.	97
	CYTISINE GROUP		
	cytisine	*Cytisus laburnum* L. (*Laburnum vulgare* J. Presl.)	47

TABLE I

Representative Quinolizidine Alkaloids

Structure	Name	Example plant	Reference
	N-methyl-cytisine	*Cytisus laburnum* L.	70
	rhombifoline	*Thermopsis rhombifolia* Richards	72
	angustifoline	*Lupinus angustifolius*	71

THE SPARTEINE / MATRINE GROUP

THE SPARTEINE FAMILY

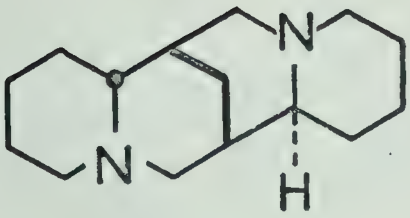
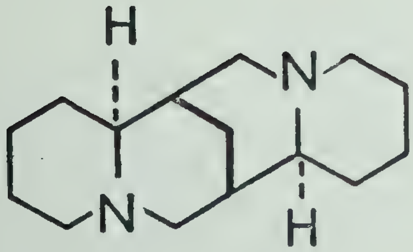
	sparteine (lupinidine)	*Lupinus luteus* L.	46, 47
	β-isosparteine (pusilline)	*Lupinus pusillus*	46, 73

TABLE I

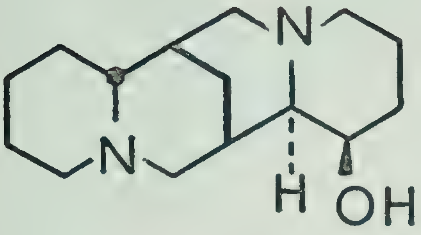
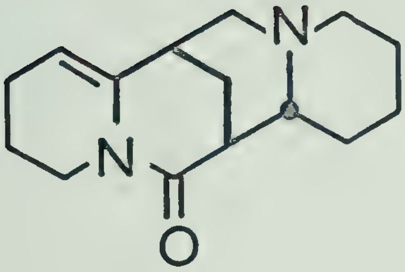
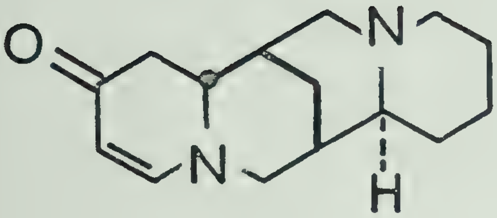
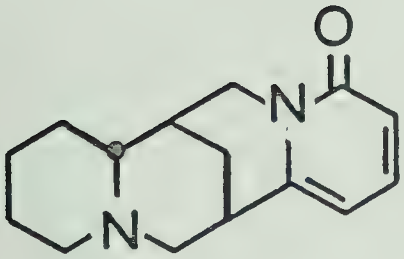
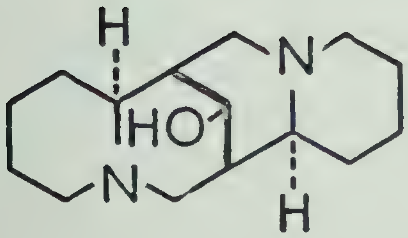
Structure	Name	Example plant	Referenc
	retamine	Retama sphaerocarpa Boiss	46, 75
	monspessulanine	Cytisus monspessulanus L.	77
	multiflorine	Lupinus multiflorus Lam.	78
	thermopsine	Thermopsis lanceolata R. Br.	47, 79
	hydroxysparta-lupine	---	46

TABLE I

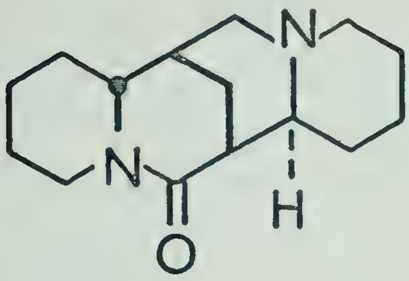
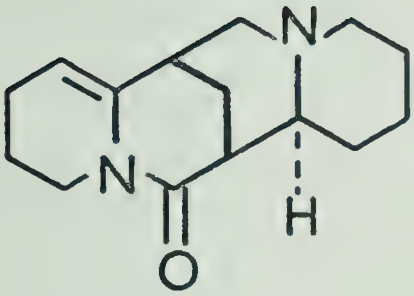
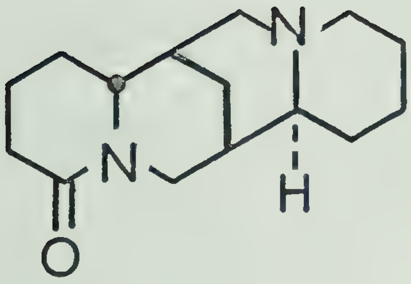
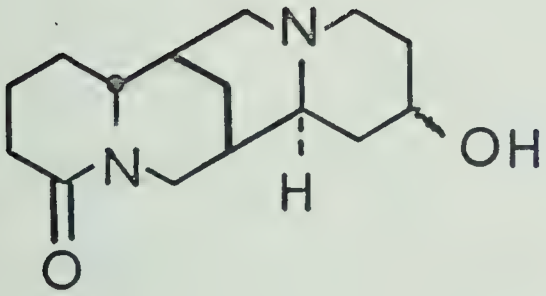
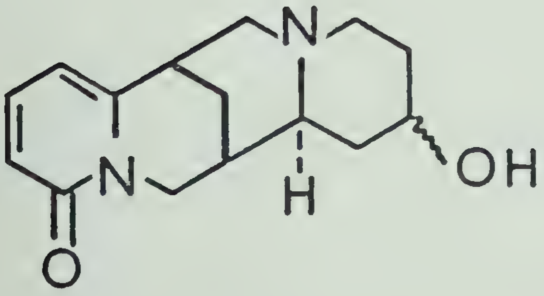
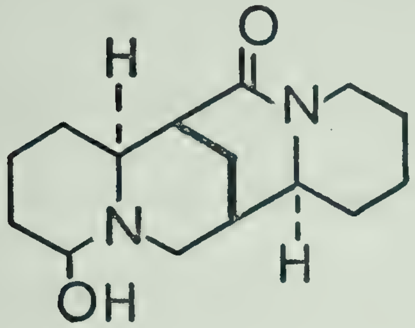
Structure	Name	Example plant	Referenc
	aphylline	*Anabasis aphylla* L.	46, 47
	aphyllidine	*Anabasis aphylla* L.	46, 47
	lupanine	*Lupinus kingii*	46, 47
	13-hydroxylupanine	*Lupinus albus* L.	46, 47
	baptifoline	*Baptisia perfoliata* L.	46, 76

TABLE I

Structure	Name	Example plant	Referenc
	lupanoline	*Lupinus sericeus* Pursh	46, 80

THE MATRINE FAMILY

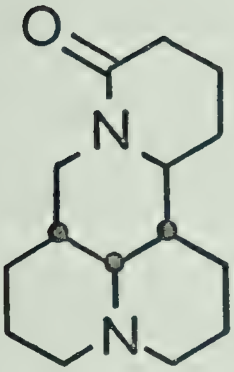
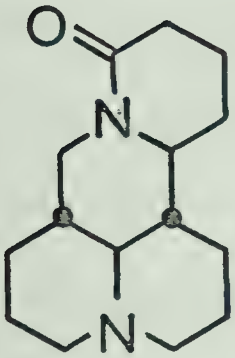
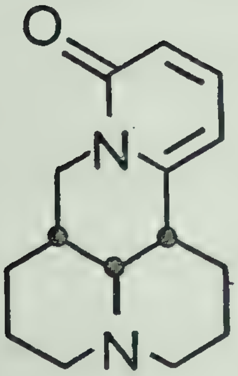
Structure	Name	Example plant	Referenc
	matrine	*Sophora flavescens*	46, 81
	allomatrine	*Leontice eversmanni*	46, 81
	sophoramine	*Sophora pachycarpa* Schrenk.	46, 81

TABLE I

There are several types of non-quinolizidine alkaloids found in quinolizidine-forming plants. These are reviewed in references 46, 47 and 82. The stereochemistry and conformation of the quinolizidine alkaloids are discussed in a later section. The many structural isomers possible in this group contributes to the large numbers of closely related quinolizidines known. Table II illustrates some of the derivatives of the quinolizidines that have recently been discovered in various plants. All of the compounds in Table II are esters of either hydroxylupanine or of lupinine with various organic acids. Alkaloid-peptides have been synthesized from cytisine (87). (Only quinolizidines related to cytisine are able to form peptide-like bonds without quaternization of the ring nitrogens). So far as can be ascertained such compounds as N-alanylcytisine and N-glycylglycylcytisine have not been found in plants. These workers (87) have done extensive work on the formation of various alkaloid-peptides but have published only in Russian and generally in a journal not readily available here. None of the abstracts (in Chemical Abstracts) regarding their work make any mention of the natural occurrence of these compounds. It is presumed that the syntheses of alkaloid-peptides have been purely chemical and indicate only the possibility of similar compounds forming in vivo.

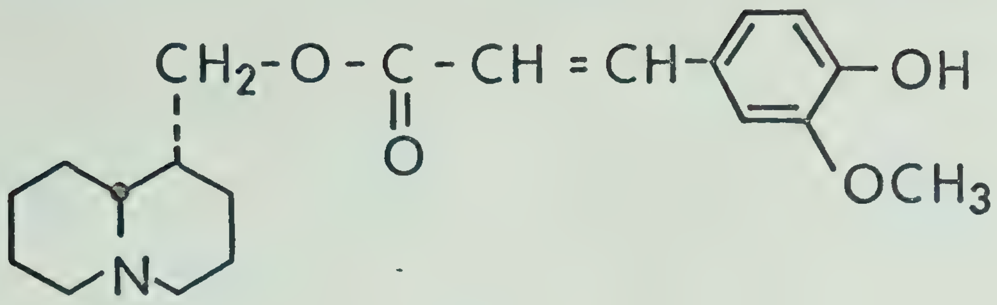
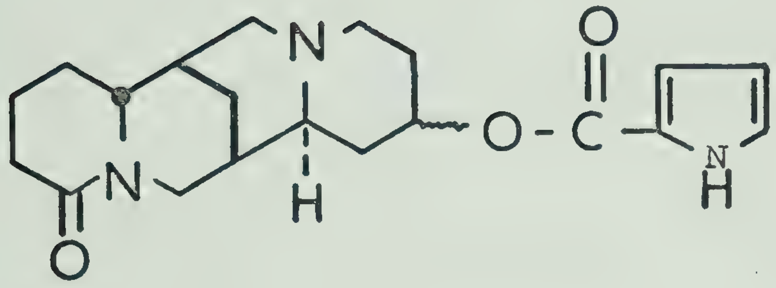
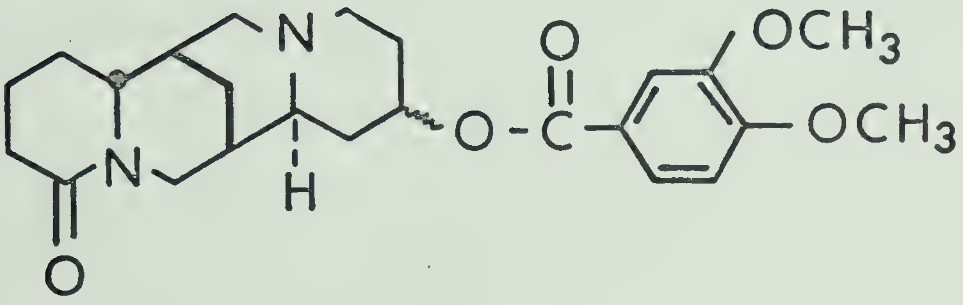
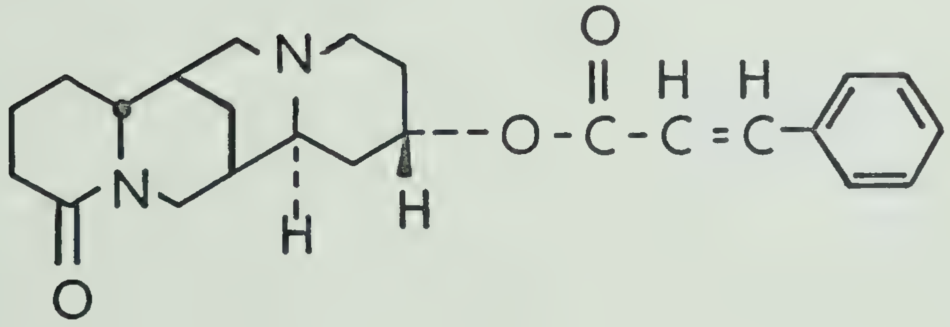
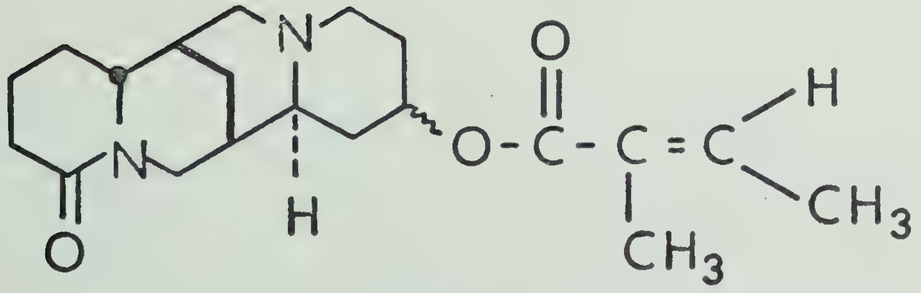
Structure	Name	Example plant	Reference
	ω-feruloyloxylupinane	*Lupinus luteus*	86
	calpurnine	*Calpurnia* subdecandra	83
	cineverine (verateoyl-β-hydroxy-lupanine)	*Genista cinerea*	84
	13-*trans*cinnamyl-hydroxylupanine (*cis* isomer also present)	*Lupinus angustifolius*	85
	13-hydroxylupanine tiglate	*Lupinus albus*	85

TABLE II. Quinolizidine Derivatives

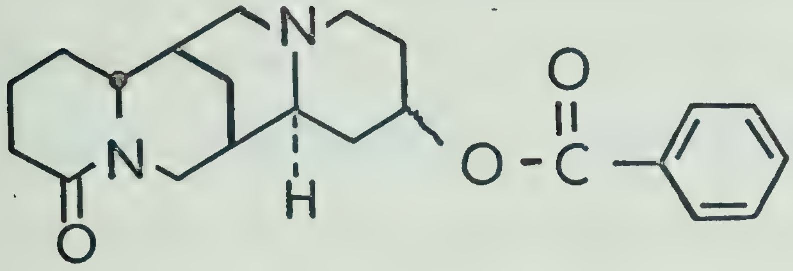
Structure	Name	Example plant	Reference
	13-hydroxylupanine benzoate	*Lupinus albus*	85

TABLE II

(2). History

Lupinine was first isolated from Lupinus luteus seeds by Cassola in 1835 and was crystallized by Baumert in 1881. Cytisine was isolated and purified by Husemann and Marme in 1862, and sparteine was isolated around 1895 by Nagai (47). Willstätter and Fourneau correctly assigned the molecular formula of lupinine in 1902. Winterfeld and Holschneider first synthesized quinolizidine in 1932 and in 1937 lupinine was synthesized by Clemo, Morgan, and Raper. The absolute configuration of lupinine was established in the early 1950s. Clemo and Raper assigned the correct structure to sparteine in 1933, and it was totally synthesized first by Leonard and Beyler in 1948. The chemistry of the quinolizidines has since been studied extensively. Leonard lists 330 references in his first review and an additional 199 in his second review prepared six years later.

(3). Pharmacology - Effects on Animal Organisms: Uses

The quinolizidine alkaloids have not been widely used in medicine. In general the quinolizidine alkaloids are mildly toxic, although cytisine is a relatively powerful poison that causes nausea, convulsions and respiratory failure. Its action is similar to that of nicotine, but cytisine is less toxic. Certain of the quinolizidines have been noted to stimulate (in low doses) and depress (high

doses) the action of isolated animal hearts, and have been used *in vivo* to produce a temporary increase in blood pressure. Sparteine is toxic and similar in action to (although less toxic than) coniine. It is a heart and circulatory depressant and paralyzes motor nerve endings and sympathetic ganglia. Sparteine, lupinine and lupanine (in that order) stimulate contractions of rabbit uterus. The minimum lethal doses (mg./kg. body weight) of lupinine, sparteine and lupanine for guinea pigs are 28-30, 42-55, and 25-29 (18).

Sparteine sulphate has been used to reduce blood loss and to accelerate delivery in some cases of pregnancy (88, 89). Sparteine also appears to enhance the analgesic action of certain drugs (*e.g.* aspirin) (90). Some derivatives of quinolizidine and of lupinine itself have been developed for use as local anaesthetic agents (18, 91). Another quinolizidine derivative has been patented as an antihistamine and as a uterus contracting agent (92).

Quinolizidine alkaloids have been also used as insecticides (47).

The pharmacology of lupanine and some of its relatives has been studied. The minimum lethal does (*i.e* dose sufficient to kill one-half of the test animals) for lupanine, sparteine, 13-hydroxylupanine, 17-oxolupanine,

lupanine-N-oxide, and 17-hydroxylupanine for mice are 159.0, 60.5, 189.0, 690, 1,780, 177 mg./kg. of body weight respectively (93, 94).

Some varieties of lupine have been used as sheep fodder in Australia. In some instances a diet of bitter lupine produces a disease known as 'lupinosis'. It is characterized by listlessness and unusual apathy followed by jaundice (95). The disease seems to be caused by a malfunction in copper metabolism - the liver accumulates toxic levels of copper (96). There is some evidence that the condition is not caused by the alkaloids in the lupine fodder (given in discussion section of reference 65).

(4). Distribution of Quinolizidines in the Plant Kingdom

The quinolizidine alkaloids are most abundant in genera of the *Dicotyledoneae* subclass family *Leguminosae* and especially in those genera from the subfamily *Papilionaceae* (18, 47). There has been one report of quinolizidine-type compounds being found in a plant of the subclass *Monocotyledoneae* in the order *Liliales* and family *Liliaceae* (100). In the *Dicotyledoneae* quinolizidines have been reported in four of the five major groups. There are six orders (out of 56 orders in this subclass) known to form these compounds. Quinolizidines occur (in addition to the *Leguminosae* family) in the families *Chenopodiaceae*, *Berberidaceae*, *Papaveraceae* (18, 47), *Solanaceae*, *Compositae*,

Ranunculaceae, Monimiaceae (15), Nymphaeaceae (99), and Scrophulariaceae (10). Table III lists all of the families and genera known to form the parent and important quinolizidine alkaloids (sparteine, lupanine, lupinine, cytisine, and matrine) and fits the alkaloid forming genuses into the general scheme of plant classification. The classification system used is that described by Benson (102). Genera were located in this structure with the help of Hutchinson's compilations of the genera of the higher plants (103, 104). An important source of the data used in Table III was the compilation of alkaloid-forming plants prepared by Willaman (15). This reference covers work done up to 1959 and has been augmented by data from references 10, 46, 47, 97, 98, 99, 100, 101, and 105. There may be other genuses forming other quinolizidine alkaloids that were not included in this survey, but this table represents at least the majority of those genuses capable of forming some and perhaps all of the quinolizidine alkaloids. This table has considerable interest with respect to the distribution, chemotaxonomy, biosynthesis and function of these alkaloids. Reference 10 contains a similar table, but employs a different classification scheme.

Chemotaxonomy of the quinolizidine and of the Leguminosae alkaloids in general has been discussed by Hegnauer (10) and Alston (19). A survey and biosystematic

TABLE III. Listing of Genera Known to Form Quinolizidine Alkaloids

subclass

MONOCOTYLEDONEAE

order

LILIALES

family	genus
Liliaceae	Schoenocaulon (100) (forms hydroxyquinolizidines)

subclass

DICOTYLEDONEAE

group

THALAMIFLORAE
(receptacle flowers)

order

RANALES

family	genus
Monimiaceae	Peumus (Sp)
Ranunculaceae	Aconitum (Sp)
Berberidaceae	Caulophyllum (MeCy) Leontice (La)
Nymphaeaceae	Nuphar (99) (deoxynupharidine)

order

PAPAVERALES

family	genus
Papaveraceae	Chelidonium (Sp)

order

CARYOPHYLLALES

family	genus
Chenopodiaceae	Anabasis (Li)

group

COROLLIFLORAE
(corolla flowers)

order

POLEMONIALES

family	genus
Solanaceae	Solanum (La)

group

CALYCIFLORAE
(cup flowers)

order

ROSALES

family

Leguminosae

subfamily	genus
Caesalpinoideae	Gleditsia (Cy)

subfamily	genus
Papilionoideae (Fabaceae, Papilionaceae, or Lotoideae)	Ammodendron (Sp) Ammothamnus (Sp) Anagyris (SpCy) Baptisia (SpCy) Cladrastis (Cy) Coronilla (Cy) Cytisus (SpCy)

genus
Euchresta (Cy)
Genista (SpCy)
Hovea (Sp)
Laburnum (Cy)
Lotus (Cy)
Lupinus (SpLiM)
Maackia (La) (101)
Ormosia (SpM) (105)
Piptanthus (SpCy)
Podalyria (La)
Retama (SpCy)
Sarothamnus (LaSp)
Sophora (SpCyM)
Spartium (SpCy)
Spartocytisus (Spartocytisine) (98)
Templetonia (Cy)
Thermopsis (SpCy)
Ulex (Cy)
Virgilia (La)

group	order	family	genus
OVARIFLORAE (ovary flowers)	ASTERALES	Compositae	Senecia (M,Cy)

Sp: sparteine, Cy: cytisine, MeCy: N-methyl cytisine,

La: lupanine, Li: lupinine, M: matrine

study of the quinolizidine alkaloids in the genus Baptisia has been reported (27). The flavonoids of Baptisia species are also of taxonomic importance. The genuses Cytisus and Genista have also been systematically surveyed for alkaloids (106, 107).

(5). The Molecular Structure and Chemical Reactions of the Quinolizidine Alkaloids

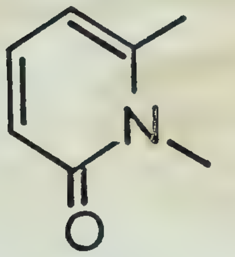
(a). introduction

The quinolizidines have been extensively studied by chemists for the past thirty years. References 46, 47, and 66 contain reviews of the chemistry of these compounds. Although there are no recent reviews on the chemical synthesis of these alkaloids, references 109 and 110, 111 and 81 contain brief review of synthetic methods for alkaloids of the lupinine family, for the sparteine group, and for the matrine group respectively. Much of the present knowledge of the quinolizidines has resulted from the studies of Bohlmann and Winterfeldt in Germany, Marion in Canada, Leonard in the United States, and Wiewiorowski in Poland.

(b). the spectra of the quinolizine alkaloids

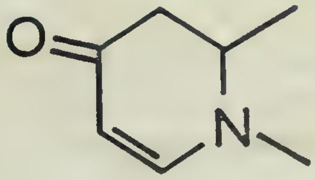
The quinolizidine alkaloids rarely absorb visible light and their spectra in the visible regions are uninformative. Many quinolizidines show absorption in the ultraviolet. Quinolizidine (in diethyl ether) absorbs

maximally at 215 mμ, and sparteine (in diethyl ether) at 214 mμ (112). The position of maximum absorption shifts towards longer wavelengths if there is a double bond adjacent to the tertiary nitrogen (*i.e.* if the compound is α - β unsaturated) because of the interaction of the unshared pair of electrons on the nitrogen with the π electrons of the double bond. A double bond more distant from the nitrogen does not have this effect (112). In the near ultraviolet only certain of the quinolizidines absorb. Alkaloids containing pyridone systems do have maxima in the 300-360 mμ range (27).



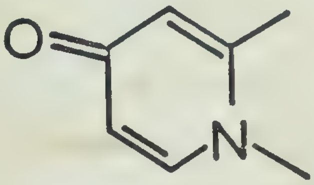
I

max. = 308 mμ, 233 mμ



II

max. = 315 mμ



III

max. = 315 mμ, 260 mμ

The α-pyridone chromophore (I) is the most common and occurs, for example, in cytisine, anagyrine, thermopsine, and baptifoline. Chromophores II and III occur in multiflorine and Δ^5-dehydromultiflorine (27).

Studies of the infra-red (IR) spectra of the quinolizidine alkaloids have been particularly fruitful, especially with respect to the conformation of these

alkaloids. Marion, Ramsay, and Jones (113) have reviewed the general IR characteristics of some of the lupine alkaloids. The region 3700-3200 cm.$^{-1}$ contains the stretching frequencies of the imino and hydroxyl groups, while the carbonyl stretching frequencies lie in the 1780-1620 cm.$^{-1}$ region. Representative infra-red studies (particularly in relation to conformation) for the matrine group are reported in reference 114, for the lupinine family in references 115 and 116, for the sparteine group in 115, 117 and 118, and for the N-oxides of the sparteine group in reference 119. The interactions of certain protons with the lone pair of electrons of the nitrogen gives rise to strong C-H stretching absorbance bands in the 2700-2800 cm.$^{-1}$ region (116). This band generally occurs when the two rings of quinolizidine are trans fused (i.e. this band occurs where two or more protons are anti to the lone pair of electrons on the nitrogen) (116). The conventions used for conformation and stereochemistry are discussed in references 120, 121, and 122. This region (or band) has been particularly useful in studies of conformation and is known variously as the 'Bohlmann region', the 'T-band', or the 'trans-band'.

Nuclear magnetic resonance (NMR) has become the most important tool for the determination of the conformations and identities of the quinolizidine alkaloids. The NMR spectra

of all of the different quinolizidine alkaloids are distinctive and correspondingly NMR is very useful in the identification of these alkaloids. NMR spectra are discussed in references 27, 123, 124, 125, 126, and 127.

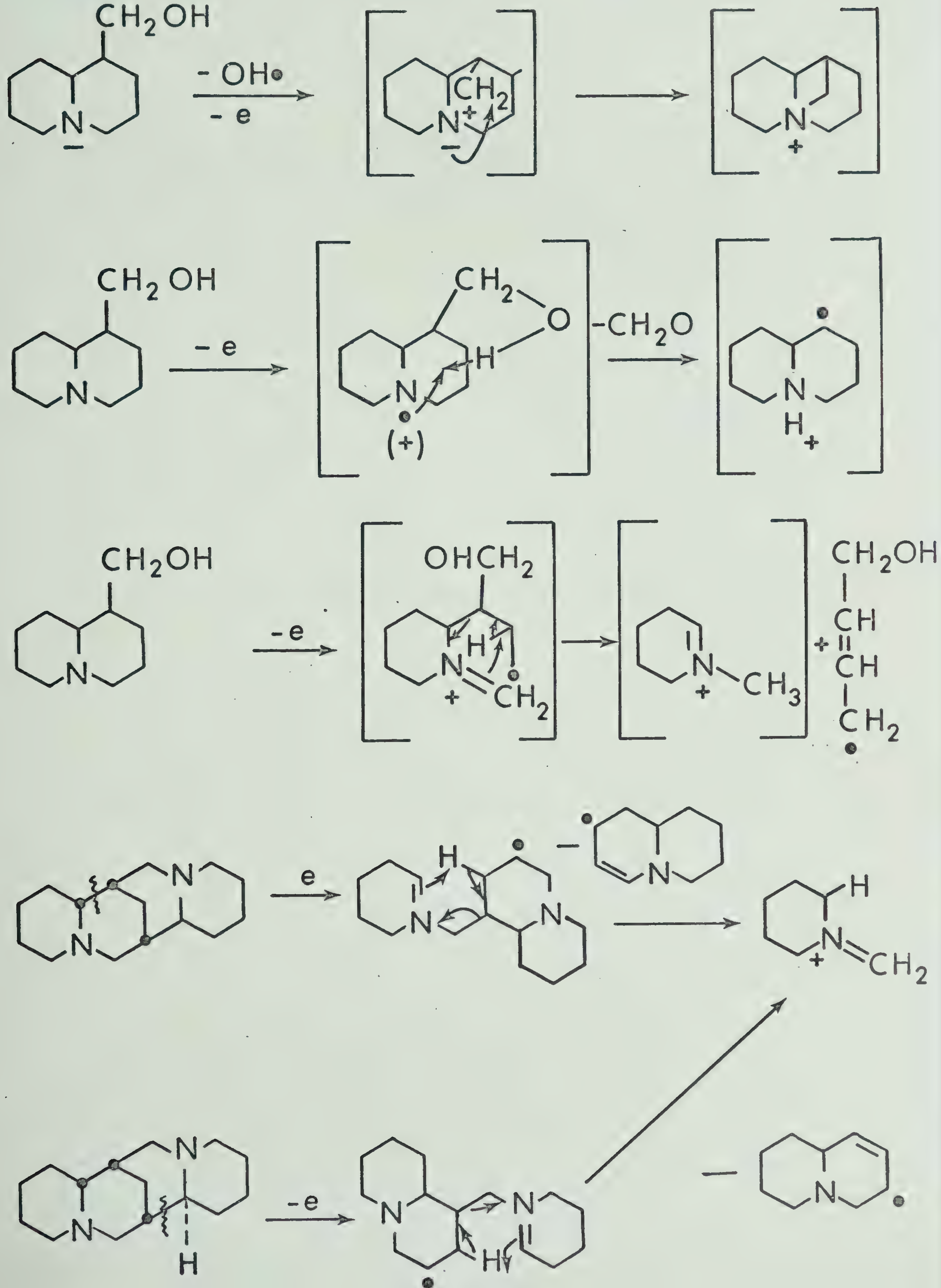
The mass spectra of many of the quinolizidine alkaloids have been examined and mechanisms have been assigned to the various fragmentation patterns obtained (128, 129). Some example fragmentation mechanisms for lupinine and sparteine are given in Figure I. The peak intensities and the fine structure of the spectra differ for the different alkaloid isomers. Thus mass spectroscopy is also a useful tool in conformation and identification studies. Sufficient data are now available for complete spectra analysis of most of the quinolizidine alkaloids.

(c). stereochemistry and conformation of the quinolizidine alkaloids

The determination of the exact three-dimensional structures of the quinolizidine alkaloids has for several years occupied organic chemists. In addition to studies using NMR, IR, and mass spectroscopy research using several other techniques has been reported. The dipole moments of lupinine-type alkaloids (130) and of the matrine group (131) have been employed in conformation determination. Optical rotatory dispersion and circular dichroic dispersion are

FIGURE I

Fragmentation Patterns of Lupinine and Sparteine



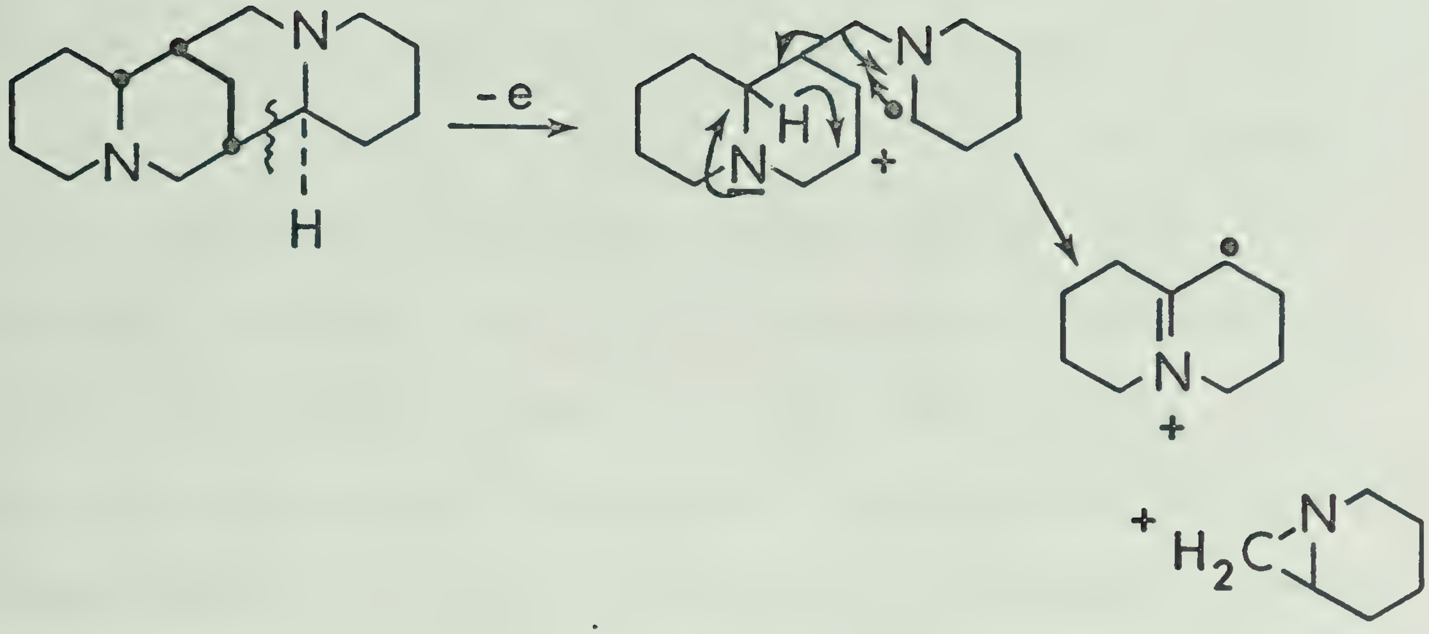
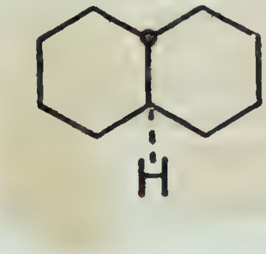


FIGURE I

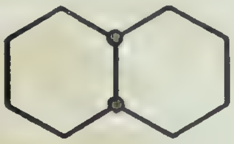
Fragmentation Patterns of Lupinine and Sparteine

also important in studies of conformation and configuration (78, 115, 132). The rates at which various related quinolizidines undergo certain reactions (*e.g.* lithium aluminum hydride reduction, solvolysis) have been used in assigning configurations (78, 115, 118, 133). The basicity of the heterocyclic nitrogens has also been related to the molecular structure (134, 135). X-ray crystallographic studies have been performed on a few members of the quinolizidine group of alkaloids. Preliminary studies on a cyanocadmium derivative of sparteine have been accomplished (136) and the complete structure of 7-hydroxy-β-*iso*sparteine perchlorate has been announced (137).

When two six-membered rings are fused together, two isomers are possible. If the two rings are so fused that the two hydrogens at the points of fusion lie on the same side of the 'plane' of the ring system, the compound resulting is the *cis* isomer. If *trans*-fusion occurs, the hydrogens at the points of ring fusion are on opposite sides of the 'plane' of the rings. Useful models for such systems are the decalins, which consist of two fused *cyclo*-hexane rings.

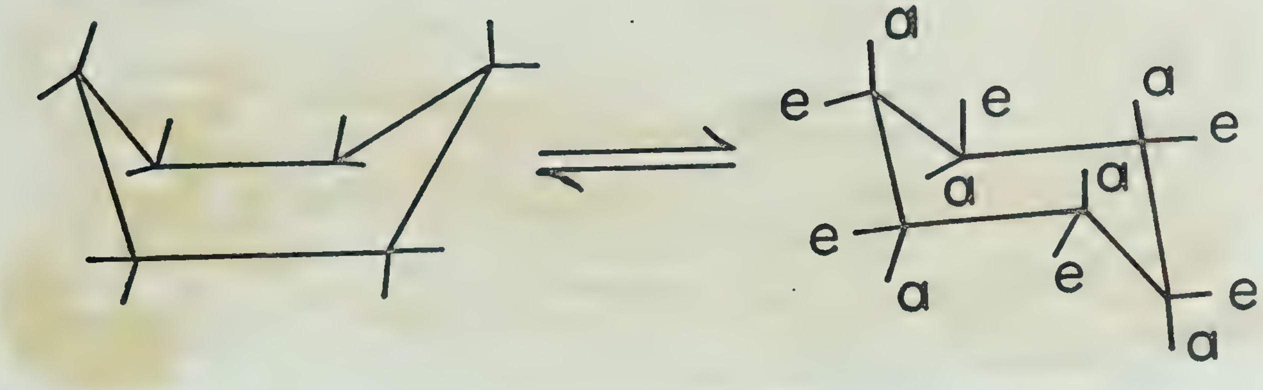


trans decalin



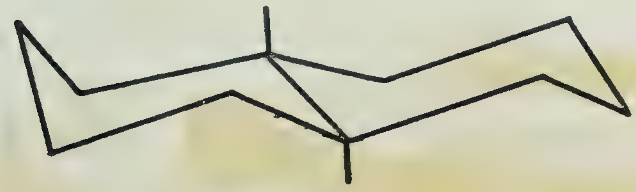
cis decalin

Cyclo-hexane commonly exists in one of two interconvertible conformations:

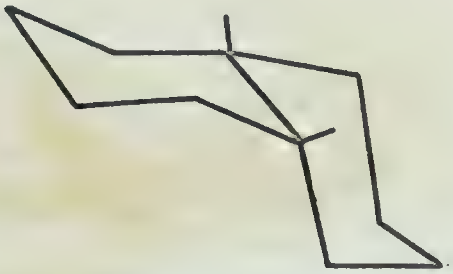


flexible form chair form

The trans juncture can be made using two equatorial (e) bonds, while the cis requires the use of one equatorial and one axial (a) bond. The most stable chair conformations for the decalins are shown below (122):



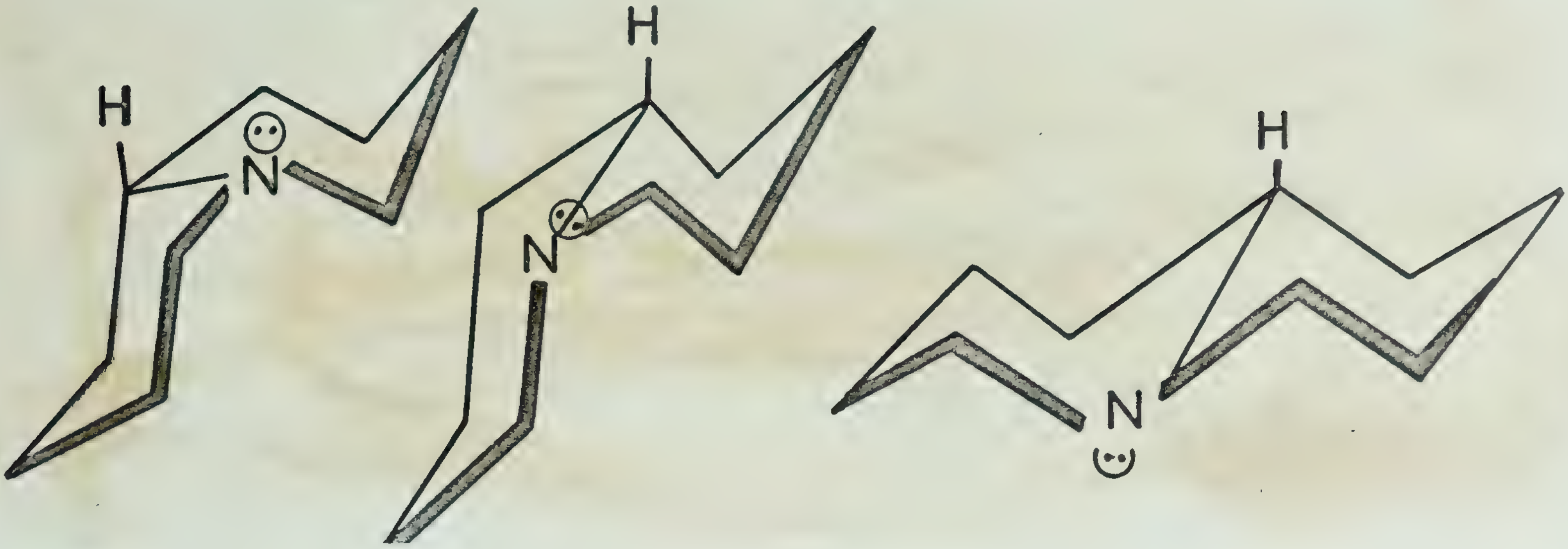
trans decalin



cis decalin

The similarities between the decalin two ring system and that of quinolizidine are apparent and the same possibilities for ring fusion exist in these heterocyclic compounds. The position of the nitrogen lone pair of electrons in quinolizidine is equivalent to the position of one of the hydrogens at the junction of the two decalin

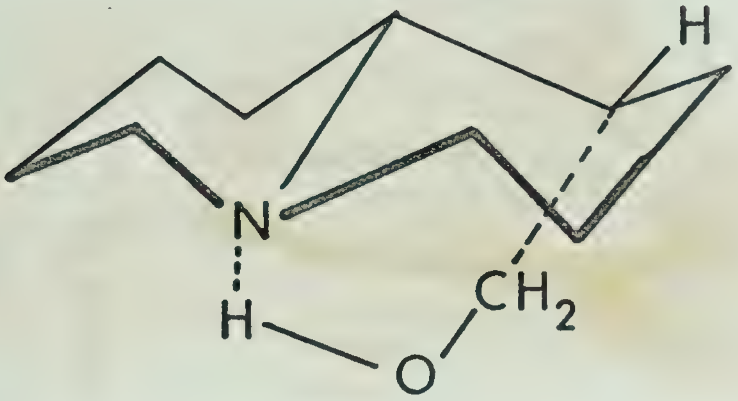
rings. Quinolizidine can exist in both a *cis* and a *trans* form (116, 125, 127).



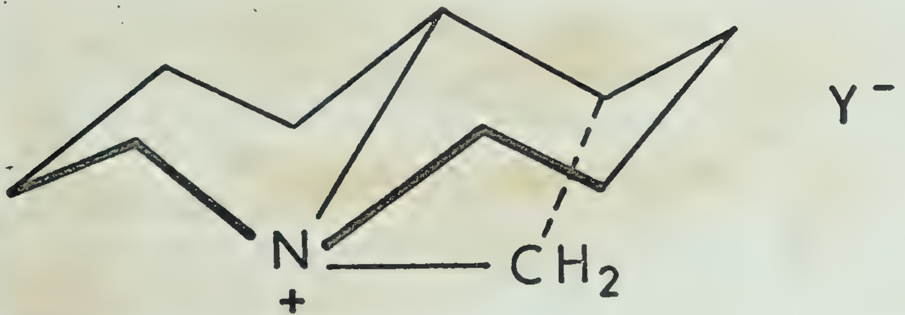
cis quinolizidine
(two views)

trans quinolizidine

It has been shown that there is no significant energy barrier to the *cis* $\rightleftharpoons$ *trans* interconversion, but the equilibrium is usually towards the more favored (fewer non-bonded interactions) *trans* form (116). *Trans* 3-hydroxyquinolizidine is favored (at equilibrium 99% is *trans*) over the *cis* form despite the possibility of hydrogen-bond formation in the *cis* isomer (116). At room temperature, better than 90% of 1-, or 2-, or 3- oxoquinolizidine exists as the *trans* form (132). In lupinine the *trans* form is greatly favored by the possibility of hydrogen bond formation (135):



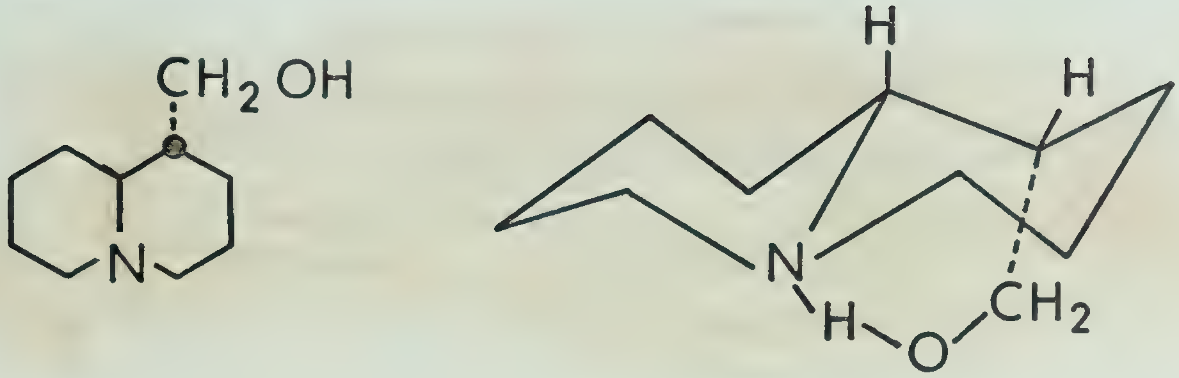
The trans conformation of lupinine permits the formation of an internal salt (126).



The conversion of lupinine to this 1,5-methylene-quinolizidinium ion requires the production of an ester derivative followed by heating. The possible existence of such a species in living plants has not been checked. The ion is reconverted to lupinine by treatment with base.

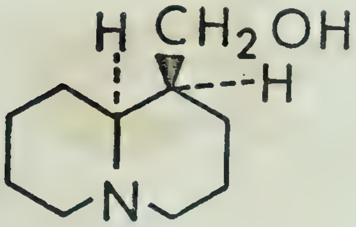
The absolute configurations of lupinine and its diastereoisomer epilupinine have been established and are

diagrammed below (46).



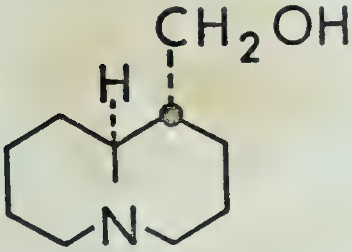
(-)-lupinine

1-α-hydroxymethyl-10-β-quinolizidine



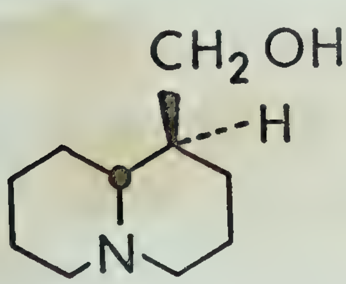
(+)-lupinine

1-β-hydroxymethyl-10-α-quinolizidine



(-)-*epi*lupinine

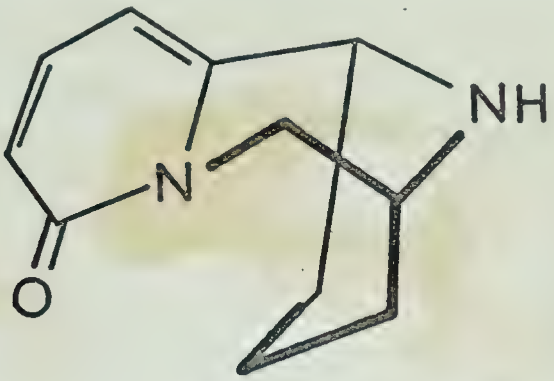
1-α-hydroxymethyl-10-α-quinolizidine



(+)-*epi*lupinine

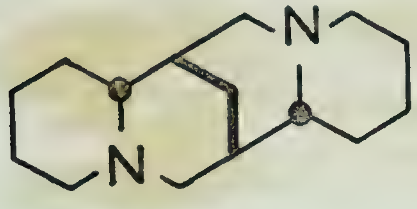
1-β-hydroxymethyl-10-β-quinolizidine

In cytisine the fusion of the third ring fixes the configurations of the two asymmetric carbon atoms with respect to each other (46). The conformation of cytisine is probably as follows (46):

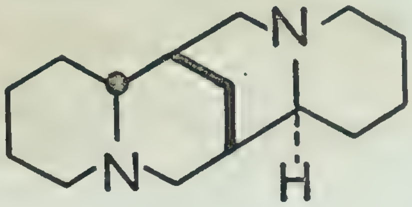


The sparteine group of tetracyclic quinolizidine alkaloids may be considered as being the result of the fusion of two quinolizidine systems. The position of the methylene bridge in these alkaloids is fixed by this fusion into a position above the 'plane' of the molecule as is shown by the darkened line used to show its position. Each of the two quinolizidine systems may be either cis or trans. This gives rise to three possible pairs of basic isomers for the unsubstituted sparteine skeleton. These are (±) cis-cis, (±) cis-trans, and (±) trans-trans. The introduction of substituents on the rings of course increases the number of possibilities. The presence of α-βunsaturation in one or both of the quinolizidine rings allows other isomers such as blank-cis and blank-trans (46). X-ray diffraction studies on (-)-α-isosparteine have shown it to be the cis-cis isomer

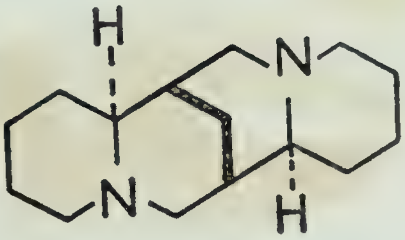
and to this known structure the other members of the family have been related (46, 137). Sparteine is *trans*-*cis* (46, 134, 138) and β-*iso*sparteine *trans*-*trans* (46, 137).



α-*iso*sparteine
(*cis*-*cis*)

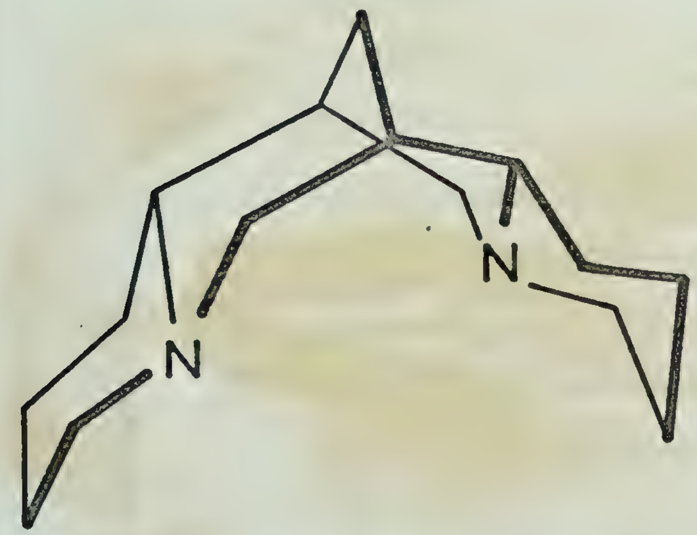


sparteine
(*cis*-*trans*)

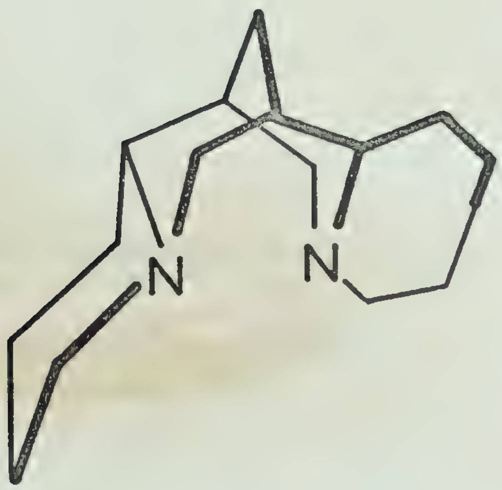


β-*iso*sparteine
(*trans*-*trans*)

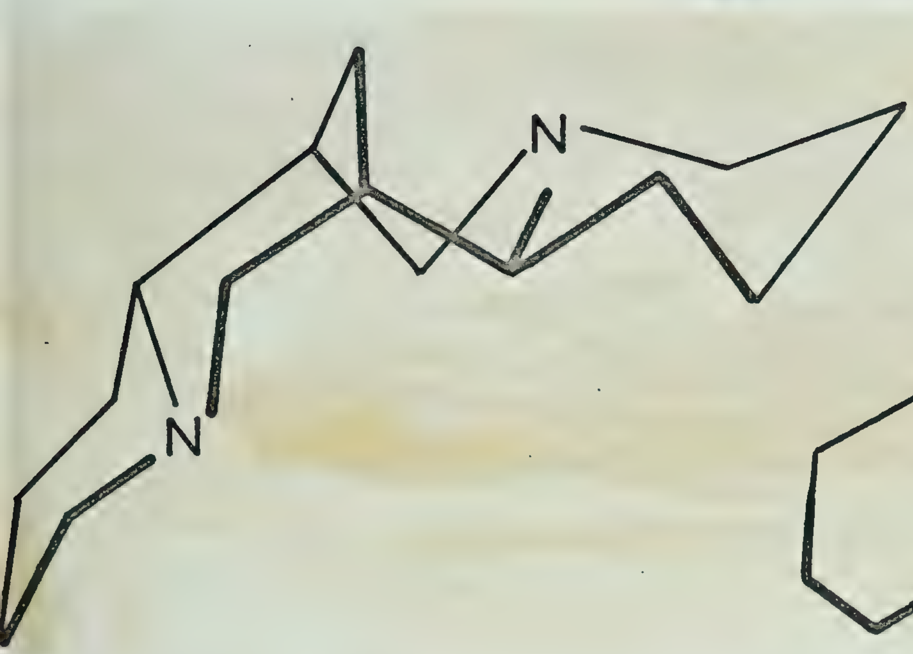
The conformations of these three alkaloids are shown below (123, 125, 138).



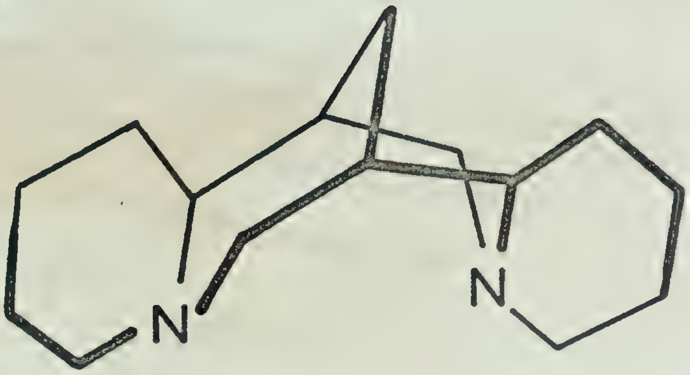
α-*iso*sparteine



sparteine (chair)



sparteine (boat)

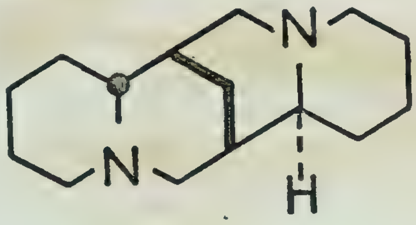


β-*iso*sparteine

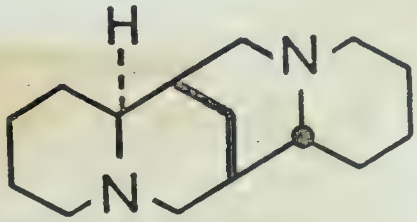
The spacing between the nitrogens in both α-*iso*sparteine and sparteine is about 3.0 Å (134), but the conformation of α-*iso*sparteine tends to allow the formation of an intramolecular hydrogen bond in the α-*iso*sparteine monocation, which stabilizes it relative to sparteine and relative to its dication and to its free base. The differing basicities of the nitrogens of the members of this group of compounds can be explained on the basis of molecular conformation (46, 123, 134, 135, 137). There is some debate as to whether sparteine exists as the chair or as the boat conformation. Recent studies indicate that although the boat form is favored in apolar solvents (125), the chair form predominates in more polar solvents (123). The interconversion between these two conformations is facile. The N-oxides of this series provide examples of very strong and symmetrical intramolecular hydrogen bonding

(119, 123). These molecules can also form strong intermolecular hydrogen bonds (119). Sparteine and α-*iso*sparteine can be interconverted by catalytic hydrogenation at temperatures above 100° (138). Reference 139 is illustrated with photographs of space-filling models of the various members of the sparteine group.

The absolute configurations of many members of the sparteine group have been established (115, 140).

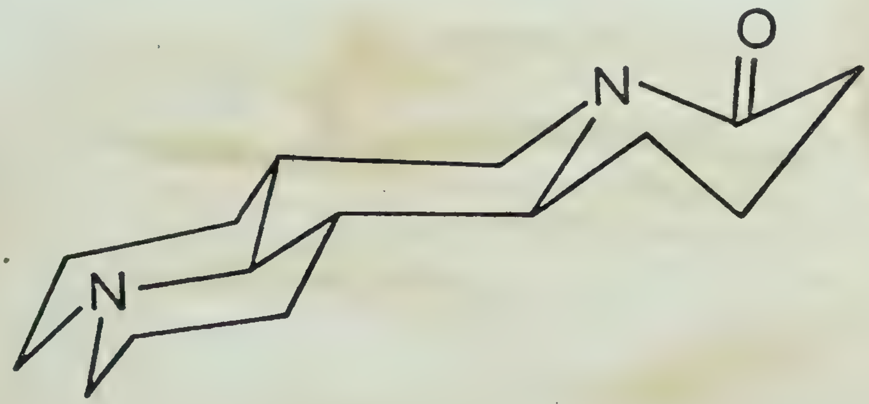


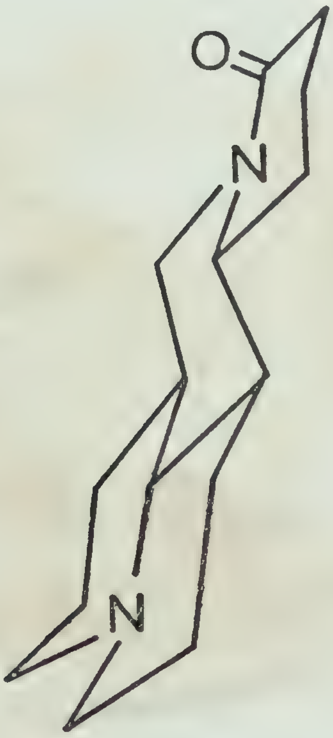
(+)-sparteine



(-)-sparteine

Alkaloids of the matrine group have two possible stable conformations (114).





The absolute configurations of some members of this group have recently been assigned (141).

(d) basicity of the quinolizidine alkaloids

Table IV lists the pK values obtained for various of the quinolizidine alkaloids. It is customary to determine the pK values in an organic solvent system such as 66% aqueous dimethyl formamide, 50% aqueous ethanol or 80% aqueous 2-methoxyethanol (46, 134). Reference 134 discusses the dissociations of the alkaloids in 2-methoxyethanol (ME) and shows that the pK values obtained in this system are equivalent to true pKa' values.

(e). some reactions of the quinolizidine alkaloids

Many of the chemical properties of the quinolizidine alkaloids have their origins in the lone pairs of electrons available on the tertiary nitrogens. The interaction of these electrons with double bonds in the system has been discussed above, as has the protonation of these nitrogens. The quinolizidine alkaloids can form complexes with various cations (particularly with transition metal cations - as is a characteristic of most alkaloids) (136, 143), and can form salts with most organic and inorganic acids.

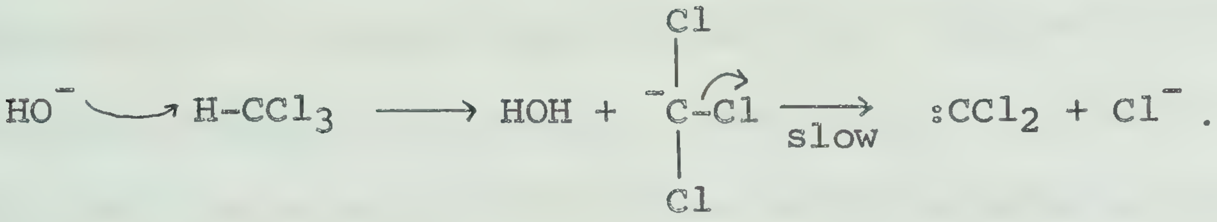
Some quinolizidine compounds (particularly when in the free base form) undergo reactions with chloroform (27, 144, 145). The nature of the reaction is not known,

Alkaloid	pK'(1)	pK'(2)	Solvent	Reference
quinolizidine	10.58	-	ME	135
lupinine	8.25	-	ME	135
sparteine	3.05	10.26	ME	135
	3.3	11.4	dimethyl formamide	46
	2.24	9.51	H_2O	142
α-isosparteine	2.61	11.26	ME	135
	<2.5	12.1	dimethyl formamide	46
lupanine	7.47	-	ME	135
α-isolupanine	7.45	-	ME	134
13-hydroxy-lupanine	7.37	-	ME	134
17-hydroxy-lupanine	9.30	-	ME	134
anagyrine	6.45	-	ME	134
multiflorine	7.60	-	ME	134
thermopsine	5.70	-	ME	134
sparteine-N_6-oxide	2.56	11.94	ME	134
α-isosparteine-N-oxide	2.48	12.27	ME	134
lupanine-N-oxide	5.91	-	ME	134

Table IV. pK' Values for Quinolizidine and some Quinolizidine Alkaloids

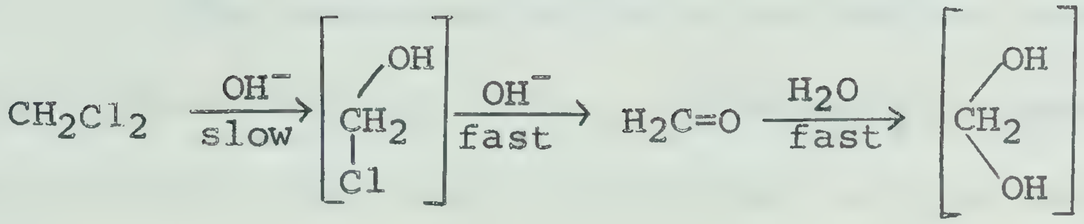
ME = 2-methoxyethanol

but it seems to be promoted under basic conditions (145) and in the presence of oxygen (27, 144). Alkaline chloroform converts 17-hydroxysparteine to 17-trichloromethylsparteine. It is possible that some of the reactive hydrolysis products of chloroform are involved. The mechanism of chloroform hydrolysis is thought to be as follows (122, 146):



$$HO^{-} \curvearrowright H\text{-}CCl_3 \longrightarrow HOH + \overset{Cl}{\underset{Cl}{{}^{-}C}}\text{-}Cl \xrightarrow[slow]{} :CCl_2 + Cl^{-}.$$

Both the trichlorocarbanion and the more stable dichlorocarbene are very reactive nucleophilic species. The dichlorocarbene generally reacts with water to form carbon monoxide, formate, and chloride ions (146). Thus, although chloroform is a standard alkaloid extractant and is often used to extract alkaloids from basic aqueous solutions, its use is hazardous and may give rise to artefacts (27, 144). Chloroform may also spontaneously be oxidized to phosgene (carbonyl chloride) (146) which is known to react with lupinine (147). Dichloromethane does not seem to undergo reaction with the alkaloids and is thus a better extraction solvent (27). Dichloromethane does undergo reaction with hydroxyl ions through a nucleophilic substitution mechanism to give chloromethanol, which then forms formaldehyde (146).

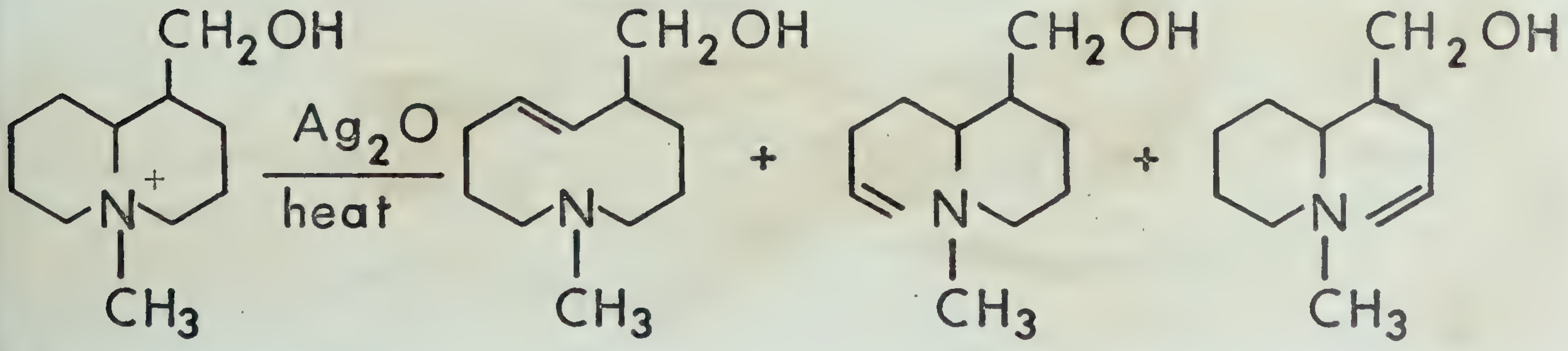


$$CH_2Cl_2 \xrightarrow[slow]{OH^-} \left[\begin{array}{l} \;\;OH \\ CH_2 \\ Cl \end{array}\right] \xrightarrow[fast]{OH^-} H_2C{=}O \xrightarrow[fast]{H_2O} \left[\begin{array}{l} \;\;OH \\ CH_2 \\ \;\;OH \end{array}\right]$$

This reaction is very much slower than that of chloroform and does not give products likely to attack alkaloids.

The decomposition of quinolizidine quaternary ammonium salts (Hofmann degradation) is a frequently used structure determination method. Naturally occurring quaternary ammonium quinolizidines could be detected by treatment of the dried residue of an aqueous plant extract with silver oxide under the conditions required for Hofmann degradation. (The permanently charged quaternary salts are not extracted by the usual procedures for alkaloid extraction). Quaternary ammonium salt-type alkaloids have been detected in several plant species (172) although lupines were not examined. The presence of typical Hofmann decomposition products would confirm the presence of quaternary alkaloids in the extract. Hofmann degradation of N-methyl lupinine can give several products (148, 149).

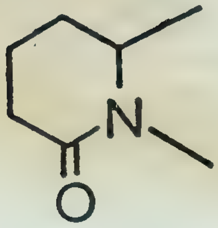
e.g.



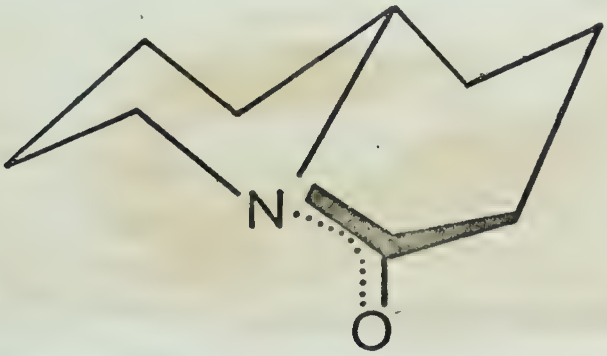
The oxidation of the quinolizidine alkaloids by oxygen has not been studied. Oxidations are known to occur (particularly when the alkaloids are in free base form) during extraction and isolation of the alkaloids (27, 144). These reactions are usually slow and are correspondingly not important during most isolation procedures if high temperatures are avoided (144). (I have found that a 1% sparteine solution in chloroform exposed to oxygen for one month became dark brown and gave at least eight different products by gas chromatography. Other quinolizidine alkaloids oxidized at very much slower rates). Jaminet (150) studied the changes occurring with time in solutions of sparteine in ethanol and chloroform. He found that sparteine underwent several reactions, some of which were light promoted, others of which seemed to be metal-ion catalyzed. Aqueous solutions were very much more stable (150). The chemical synthesis of quinolizidine alkaloid N-oxides requires quite extreme conditions, although many N-oxides are also formed biologically (46). Sparteine is converted to its N-oxide by refluxing for twenty hours in 5% hydrogen peroxide (151). Some of the more common chemical oxidations of the quinolizidines are discussed by Leonard in references 46 and 47.

Those quinolizidine alkaloids that have a carbonyl group adjacent to one of the nitrogens are lactams or 'inner

amides' (for example, lupanine, monspessulanine, aphylline, matrine).

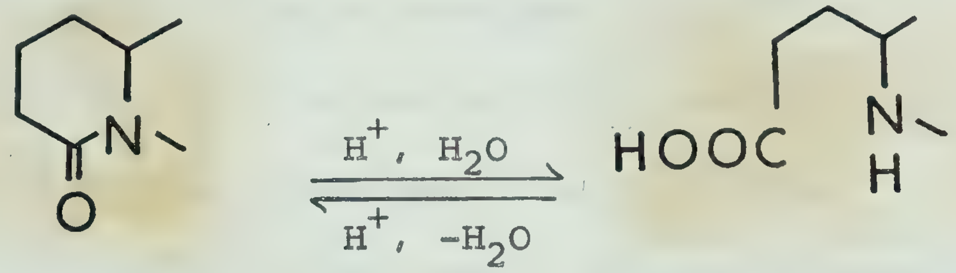


The lactam-containing quinolizidine alkaloids undergo some reactions characteristic of lactams. Some of the reactions of the lactam alkaloids are discussed in references 133 and 139. The position of the lactam grouping in the molecule greatly affects its reactivity. Some lactams are very reactive; others very stable, depending upon the molecular conformation (133). The electrons of the nitrogen can resonance-interact with the carbonyl group. An NMR study of a model compound (quinolizidone) showed that the electrons are delocalized between the nitrogen and carbonyl group (124).



Ionization of the nitrogen removes the possibility of this interaction. Probably for this reason the lactam alkaloids show only one ionizable nitrogen (cf. Table IV). When the pH is sufficiently low to protonate the nitrogen, the lactam

will hydrolyse. Lupanine hydrolyses in both acid and base. Between pH 0 and 11 lupanine exists in equilibrium with lupaninic acid (152).



At equilibrium at 35° in 0.2N, 5N, and 10N HCl the following amounts of lupanine were converted to lupaninic acid respectively: 11.8%, 88.7%, and 0%. The absence of hydrolysis in 10N HCl was explained by a lack of 'free water' at this acid level. The acid hydrolysis reached equilibrium in about fifty hours. The basic hydrolysis was slower than the acid hydrolysis of lupanine and at least sixty hours at 50° were required for equilibrium to be obtained, however the conversion was better. Ninety percent of the lupanine was hydrolyzed by 1N KOH and 15% by 0.01N KOH (152). It is apparent that any alkaloid isolation methods that expose the lactam-quinolizidine alkaloids to highly acidic or basic conditions may result in the loss of these alkaloids (27).

(6). Isolation and Separation of the Quinolizidine Alkaloids

Many variations of the classical procedures for alkaloid extraction have been employed in the isolation of the quinolizidine alkaloids. The general procedure is to

extract dried materials (which are often moistened with aqueous ammonia or ground dry with a strong base) with various organic solvents - petroleum ether, chloroform, diethyl ether, alcohols, acetone, etc. Quinolizidine alkaloids are often extracted from the organic extracts with aqueous acid solutions. The resulting mixtures of these alkaloids are resolved by fractional crystallizations and/or distillations, and by further extractions. Such methods are rarely quantitative and may cause chemical reactions which alter the alkaloids. Leonard (46) gives some examples of the traditional extraction techniques.

An extraction method for use in biochemical studies of alkaloids must effect quantitative extraction of the alkaloids from small amounts of preferably fresh tissue without causing any changes in the alkaloids. The reactions of chloroform with the quinolizidine alkaloids have been discussed and it is apparent that chloroform should be avoided - particularly long exposures to chloroform and air must be avoided. Exposure to strong acids and bases for any but the shortest times must not be permitted lest the lactam-type quinolizidine alkaloids be lost. Criteria for extraction and isolation procedures for the quinolizidine alkaloids are discussed by Graham (7, 16) and by Cranmer (27).

Extraction of quinolizidine alkaloids from Thermopsis was found to be accomplished most effectively with methanolic acetic acid (153). Methanol alone has also been used to effect the quantitative extraction of alkaloids from lupine (144, 157). Chloroform extraction of plant materials ground dry with strong bases is still frequently employed despite the availability of gentler methods (155, 156). Some workers have subjected the chloroform - extracted substances to high vacuum distillation and to steam distillation (154). Quinolizidine alkaloids have also been removed from plant tissues by treatment with ultrasound (158) and by electrodialysis (159). These methods have not come into common use. Although the methods discussed in this paragraph are gentler than many of the earlier, classical procedures, they still do not fulfill all of the criteria of a biochemically useful extraction.

Cranmer developed an improved method that employed extraction of mildly basified, dried tissues with dichloromethane, followed by reëxtraction into dilute citric acid, and then by an extraction back into dichloromethane (27). This procedure avoided strong acids and bases as well as chloroform.

Sebyła (160) pressed frozen lupine tissues with a hydraulic press and then washed the residue with water. The

pH of the resultant extract was adjusted and the proteins were precipitated by heating. The water was removed in vacuo to leave an alkaloid residue that was purified by repeated chloroform extractions and reëxtractions into dilute HCl. The initial extraction procedure was sufficiently gentle but the following steps might damage the alkaloids.

Graham (7, 161) studied the efficiency of various methods for the extraction of quinolizidine alkaloids from fresh or frozen lupine tissue. He found water gave quantitative alkaloid extraction. The use of phosphate buffers resulted in nearly 90% extraction, dilute (0.1M) HCl or H_3PO_4 gave 70-80% extraction, and 1M H_3PO_4 extracted only 40% of the alkaloids. Graham removed the alkaloids from the aqueous extract with cation exchange resin.

The use of ion exchange resins in the isolation and separation of alkaloids is not a recent innovation. Sulphonic acid type cation exchange resins have been utilized for alkaloid isolation (162, 163, 164), but they bind quinolizidine alkaloids too strongly for quantitative use (7, 157, 161). Carboxylic acid type cation exchange resins (157) and phosphonic acid type resins (7, 161) have been successfully used for quantitative removal of quinolizidine alkaloids from aqueous solutions. Frequently the alkaloids are removed from the resin by treatment with alcoholic

ammonia solutions (157). Other workers prefer to displace the alkaloid with strong acid (7, 164). The possibility of alkaloid hydrolysis exists with both methods, but if the times of exposure are brief there is little danger of significant alkaloid loss.

Once the alkaloids have been isolated and separated from the bulk of the non-alkaloid materials, they must be separated from one another and analyzed. The problems of alkaloid loss and alteration are present in these steps as well as in the isolation and extraction procedures. Column chromatography on various absorbents has been the traditional method for resolving mixtures of quinolizidine alkaloids into their components (46, 47). Chromatography on cellulose powder columns has also been used to separate mixtures of these alkaloids (144) and is particularly useful in studies of the hydroxylupanine ester alkaloids (methanol extracted) (165). Strong aqueous acids or bases would not only slowly hydrolyse the lactam ring of the alkaloids, but would also hydrolyse the ester link.

Paper partition chromatography has seen extensive use in separating quinolizidine alkaloids. Reference 166 contains a comprehensive review of the paper chromatography of alkaloids plus a succinct summation of the solvent systems used for the quinolizidine alkaloids. E.P. White

has developed useful techniques for the chromatography of the leguminosae alkaloids on filter-paper discs (167). Paper electrophoresis is also useful in separating these compounds from one another (168). Counter-current extraction has also been recently employed (169).

Thin-layer chromatography (TLC) has supplanted paper chromatography for many applications as it is faster, more sensitive and often gives better resolution. TLC methods for alkaloid separation in general are reviewed in references 170, 171, 172, 173. Silica gel is the most frequently employed adsorbent and alkaloids are detected either with an iodoplatinate reagent or with some variation of Dragendorff's reagent (170, 171, 172, 173). The activity of this reagent is probably the complex salt $BiI_3 \cdot 4KI$ (174) which forms insoluble coloured addition products by reaction with the alkaloid heterocyclic nitrogen (175). Although Dragendorff reagent does not react with amines or amino acids, it does react with certain non-nitrogenous substances (172, 175, 176). To react with this reagent, a non-nitrogenous compound must have a conjugated carbonyl (aldehyde or ketone) group, or a lactone ring (175). Ammonia reacts with acetone to give Dragendorff positive compounds (176). Quaternary ammonia compounds also react with this reagent (172, 177).

Dragendorff reagent, however, remains the most useful and specific of the available alkaloid reagents (172) and stains quinolizidine alkaloids well (7, 174).

Thin-layer chromatographic methods for the quinolizidine alkaloids are discussed by Paris and Paris (178), Ramaut (179), Gill (180), Bernasconi, Gill and Steinegger (181), Faugeras and Paris (182)(who also used thin-layer electrophoresis), and by Cranmer (27). Most workers have used silica gel and either neutral (27) (e.g. methanol in ethyl acetate) or basic (181) (e.g. cyclohexane-diethyl amine) solvent systems.

Gas chromatography is important in both the separation and determination of alkaloids and is discussed in the next section.

(7). Quantitative Determination of Quinolizidine Alkaloids

At various times and for different purposes a vast number of analytical methods have been used in the determination of the quinolizidine alkaloids. Methods range from the weighing of a crude alkaloid extract to modern electroanalytical techniques. Higuchi and Bodin (183) have prepared an excellent and extensive review of methods available up to 1961. It deals with the majority of the techniques that have been applied to alkaloid analysis and also has useful discussions of paper

chromatography and ion exchange methods of alkaloid separation. A more recent review is that of Graf (184) who discusses the recent changes in the methods reviewed by Higuchi and Bodin, and deals with some additional techniques such as thermometric titrations, gas and thin-layer chromatography, and biological and biochemical assays. Clarke (185, 186) has described microchemical tests for large numbers of different alkaloids. Such precipitation tests often form the basis for quantitative determination methods. Most of the techniques described in these reviews have been applied to the quinolizidine alkaloids.

Some of the quinolizidine alkaloids have been determined by iodometric titration (187), by the formation of cadmium complexes (188), by ultraviolet absorption (189), by photometric titration using methylene blue (190), by a highly sensitive colorimetric procedure based upon the reaction of quinolizidine alkaloids with methyl orange (191), by planimetry or densitometry of paper chromatograms (192), by microbiological assay using a lupanine-metabolizing Pseudomonad (193), by direct or indirect titration (194), by polarographic methods (195), and by oscillopolarography (196). This listing is not complete, but gives examples of the more recently developed methods. Most of these procedures are sensitive to at least 10 μg. of alkaloid and most are accurate to within 5%.

Reifer and Niziołek (197) introduced a particularly sensitive and convenient turbidimetric (198) procedure for the microdetermination of quinolizidine alkaloids that was based on the formation of a colloidal complex between the alkaloid nitrogens and iodine in a bromide-iodide solution under acid conditions. Graham and Spencer improved the method slightly and coupled it with the gentle water extraction and ion exchange procedure described above. The ion exchange procedure was followed by extraction of the alkaloids into chloroform, concentration, and separation by paper chromatography (161). Alkaloids were eluted from the paper and diluted for analysis. The technique permits measurement of quinolizidine alkaloid levels down to 5×10^{-8} moles (161) with a maximum error of 3% (197). Besides being time-consuming, this method has some other disadvantages. The colloidal complex is unstable above 5°, is slightly soluble in the reaction mixture employed even at ice temperature, and tends to agglomerate at high alkaloid levels (197); all are factors that tend to increase the variability of the method. The yield of light-scattering complex varies with the alkaloid and the method is less sensitive for some alkaloids (161, 197). The determination (as described in references 161 and 197) is based upon measurement of the reduction of light intensity caused by the

light scattered from the colloidal product. It could probably be rendered more sensitive by direct measurement of the light scattered. Roth (199) has reviewed the principles of such nephelometric measurements as applied to biological microchemistry.

The turbidimetric procedure described above is not suited for the analysis of large numbers of samples. For biochemical studies of the lupine quinolizidine alkaloids a method that was less time-consuming and that was more suitable for accurate and dependable routine analysis of plant extracts was desirable. Gas-liquid chromatography (GLC) permits both the separation and the quantitative determination of many compounds including alkaloids (200). The techniques available for alkaloid separation and analysis by GLC are thus of particular interest. References to GLC methods for alkaloid separation are given below followed by a summation of the conditions most commonly employed. The few quantitative GLC alkaloid studies are reviewed and finally a complete review of all of the reports of quinolizidine alkaloid separation is given.

Gas chromatography of polar materials such as alkaloids is often difficult. Some alkaloids decompose very readily on the column (heated glass wool was found to catalyze the decomposition of some (202)). Metal ion

contaminants in the column packing may also cause destruction of alkaloids. Basic substances may interact with polar sites in the column packing material and this causes 'tailing' and asymmetrical peaks (203). Burchfield and Storrs (200) discuss gas chromatography in general and include a section on alkaloid separation. Fales and Pisano (203) have prepared a more comprehensive review of the problems involved in alkaloid GLC and the methods used to solve them. Applied Science Laboratories have made available a useful bibliography of gas chromatographic methods for steroids, alkaloids, and amino acids (204). In 1960 Lloyd, Fales, Highet, VandenHeuvel and Wildman introduced gas chromatography to the study of alkaloids in general by reporting the gas chromatographic behavior of forty-five alkaloids of several different classes (201). Gas chromatography is also applicable to the separation of certain quaternary ammonium compounds (216). References 184, 202, 205, 206, 207, 208, 209, and 210 give general discussions of alkaloid separation and analysis by GLC and are also useful sources for the techniques involved. A rapid screening GLC method has recently been developed to study alkaloid production by bacterial cultures (215).

Solutions (usually 0.5% to 1% w/v) of alkaloid free bases in such organic solvents as acetone, methanol and chloroform are most commonly injected (usually 0.5 to 3

μl. are injected) into the gas chromatograph (200, 201, 203). Alkaloid salts in solution have been injected directly and decompose to the free base during passage through the column (202). Other workers include a strong base in the packing at the top of the column; it serves to convert alkaloid salts to the free base forms (213). Glass columns and injection areas are commonly used to avoid alkaloid decomposition on hot metal surfaces. Most workers have employed highly purified diatomaceous earth supports, which are generally treated with a silane derivative such as dichlorodimethylsilane to block polar groups and thereby to reduce peak tailing ('silanization'). The support is usually coated with a thin layer (0.5 to 5% w/w) of a silicone polymer stationary phase. Non-silicone stationary phases are seldom used. Some workers have reported success with coated capillary columns (208). Separations are often isothermal although temperature programming has been found superior in some cases (202, 207, 210, 211). (Harris and Habgood (212) discuss the applications and advantages of temperature programming in gas chromatography). Highly sensitive ionization detectors (hydrogen flame or argon ionization) are most frequently used for detection of materials separated on the columns. Preparative methods of alkaloid separation and collection are available (27, 211).

Methods for collection of the compounds separated by gas chromatography are reviewed by Karmen (214).

Some work has been done on the use of GLC for quantitative alkaloid determination in addition to that on separation and identification. Quin and Pappas (217) determined tobacco alkaloids down to 25 μg. levels using a calibration curve based upon peak height although peak area is more frequently used. They injected 50 μl. samples and used heavily coated columns. Beckett and Triggs (218) studied the extraction of nicotine from urine and were able to quantitatively detect this alkaloid in levels as low as one nanogram. They added a related alkaloid as an internal standard for quantitation. The internal standardization method was employed by Schmerzler, Yu, Hewitt, and Greenblatt (219) to determine nanogram levels of opium alkaloids in serum and urine. Gas chromatography was used by Büchi, Iconomou, and Perlia (220) in a kinetic study of the hydrolysis of some cinchocaine homogogues. McMartin and Street (209) discuss some of the factors that affect the minimum quantities of alkaloids that are detectable by GLC.

Quinolizidine alkaloids have been little studied by gas chromatography. Lloyd _et al._ reported retention times for several quinolizidine alkaloids on diatomaceous earth columns coated with 2 to 3% of a methylsilicone polymer,

G.E.-SE-30 (201). Faugeras and Paris (221) in 1964 used gas chromatography to study cytisine, methylcytisine and anagyrine in Genista species. They employed columns filled with diatomaceous earth support coated with 10% SE-30 as well as G.E.-XE-60 (a copolymer of dimethylsilonane and cyanoethylmethylsilicone polymer), and G.E.-XF-1150 (a cyanoethylsilicone polymer). These workers have more recently reported separations of other quinolizidine alkaloids by means of 5% coatings of SE-30 and G.E.-SE-52 (a methylphenylsilicone polymer) on diatomaceous earth supports (182). Cranmer (27) and Cranmer and Mabry (223) used both analytical and preparative gas chromatography in their studies of Baptisia alkaloids. They made use of various stationary phases (from 3% to 10% w/w) coated on silanized diatomaceous earth supports. Retention data for eleven quinolizidine alkaloids on XE-60, SE-30, D.C.-QF-1 (a trifluoropropylmethylsilicone polymer), and NGS (neopentyl glycol succinate polymer) are reported. Gas chromatography has also been used to identify the hydrogenated products of the Hofmann degradation of some quinolizidine alkaloids (148). (Work has been published on the gas chromatographic characterization of the related pyrrolizidine alkaloids (222)).

D. THE GENUS LUPINUS: ONTOGENY AND PHYSIOLOGY OF QUINOLIZIDINE ALKALOIDS IN LUPINES

(1). Introduction to the Genus

(a). classification and description

The genus Lupinus belongs to the subfamily Papilionaceae of the Leguminosae family of the order Rosales, group Calyciflorae (cup flowers) of the Dicotyledoneae (72). (In one of the older classification systems the genus Lupinus is in the Genisteae tribe of the Fabaceae (Papilionaceae) family and the Leguminosae order (128).) The genus contains between 100 (103) and 300 or more (224) species. Most members of the genus are shrubs or herbs and have leaves characteristically divided into a number of leaflets (103). The flowers are the familiar 'butterfly-shaped' (hence Papilionaceae) flowers of the pea family and are borne in showy racemes (103, 224). Flowers may be blue, violet, variegated, white or yellow (103). The seeds are contained in pods (which release the seeds forcefully when ripe) and are without endosperm (224). The genus contains both annual and perennial members. Most species of the genus are able to carry out fixation of atmospheric nitrogen by virtue of symbiotic bacteria in the root nodules. The genus Lupinus is the subject of a monograph by Hanelt (225).

(b). distribution

Lupines are native to North and South America, to Southern Europe and the Mediterranean region, to tropical Africa, and to Australia and New Zealand (no clear distinction between being native and being early introduced has been made in the latter two locales, however) (65, 103, 167). North America has the largest number of lupine species (224). *Lupinus havardii* is the state flower of Texas. The distribution of the European lupine species is detailed by Hanelt (225).

(2). Uses of Lupine Plants

(a). nutritional value

Lupines, like many other legumes, are potentially useful food sources. Lupine plants have higher levels of *proto*-vitamin A, fats and proteins than some other common legumes (226). Lupine seeds contain sufficient fat to cause difficulty when some methods of alkaloid extraction are used (157). Many studies have been done on the amino acid composition of lupine seeds - both protein amino acids and free amino acids. One of the most recent papers concludes that lupine proteins contain useful amounts of all of the amino acids essential for human nutrition with the exception of methionine, which is present in too low levels (227). As the seeds may contain up to 50% by weight protein (228)

considerable interest has been centered around the use of lupines for human and animal nutrition. It is unfortunate that lupine seeds are generally high in quinolizidine alkaloids.

Sheep lupinosis has already been discussed. The effect of a lupine diet in disturbing the trace metal metabolism of sheep may not be related to the alkaloid content (65) but is certainly caused by some factor in plants of certain lupine species (229).

Reports of the toxicity of the quinolizidine alkaloids vary widely (230) but the concensus seems to be that they are not very toxic. Most workers conclude that although lupines are useful as fodder for cattle, they are not suitable for human consumption (231, 232). One worker took advantage of the bitterness of lupine seeds in an unsuccessful attempt to roast them into a coffee substitute (233)!

Low-alkaloid lupine varieties are used as animal feed in Russia, Europe, and Australia (65). Lupines are grown as 'green manure' in the United States and Europe (224, 230). Lupines are also wide-spread as decorative garden plants (103).

(3). Species and Varieties of Lupines

Studies on quinolizidine alkaloid biosynthesis in lupines have almost exclusively been carried out with varieties of just three species: *Lupinus luteus*, *L. albus*, and *L. angustifolius*. *Lupinus polyphyllus* and *L. mutabilis* have been studied on rare occasions. The alkaloid level of a particular variety is subject to changes with environmental conditions. The climate of the region may even affect the spectrum of detectable alkaloids (234). Some examples of the effect of age of the plant and its nutrition are discussed in a later section. The major alkaloids in most species of *Lupinus* are lupinine and sparteine (235, 236, 237).

Lupinus luteus is known to accumulate (-)-lupinine and *l*-sparteine (46, 47, 236, 238, 239, 240). Gramine (a non-quinolizidine protoalkaloid) is also present in the yellow lupine (241, 242). Unknown alkaloids have been reported by some workers to be present in some varieties of this species (238, 239, 240) and hydroxylupanine esters and very labile unsaturated alkaloids have been found (243). One worker (240) found lupanine to be present in a Dutch fodder variety and in a wild form, but later studies (for *e.g.* 243) have not confirmed this. (The extraction and isolation method used (240) was unusual and might have prevented lupanine hydrolysis, but might also have oxidized

sparteine to lupanine). Lupinus angustifolius elaborates at least seven alkaloids (238, 243). Hydroxylupanine, hydroxylupanine esters, lupinine, lupanine (both d and dl), angustifoline, oxolupanine, and some unidentified trace alkaloids (46, 47, 238, 243, 244, 245, 246, 247) have been detected in varieties of this blue-flowered species. Lupinus albus is known to form angustifoline, sparteine, lupanine, hydroxylupanine, hydroxylupanine esters, dehydroalbine, N-methylalbine and some unidentified trace alkaloids (238, 243, 248, 249, 250, 251, 252). Gramine has not been detected in either L. angustifolius or L. albus (241). Alkaloids present in other species are listed in references 46, 47, 238 and 253. The number of alkaloids found in a particular species often varies with the methods used for isolation and identification and there are thus many examples of contradictory results in the literature. Alkaloid levels differ widely among varieties of a species thus adding to the confusion regarding the ability of that species to form certain quinolizidine alkaloids. The above listings, therefore, must be considered as tentative and subject to revision.

High and low alkaloid varieties are found in many lupine species. Although some lupine varieties are known as 'alkaloid-free' varieties, this is a misnomer as they do accumulate alkaloids but in relatively low amounts when

compared to the high alkaloid, or 'bitter' varieties (244, 254). Better terms are 'sweet' or 'fodder' lupines rather than alkaloid-free. Typically a bitter variety may have an alkaloid content of 1.2 to 3.4% of the plant's dry weight while a sweet variety may have only 0.01 to 0.4% alkaloid (255). The wild forms of lupine species are generally bitter and the fodder types have been developed by selective breeding (240). An investigation of wild *Lupinus digitatus* was undertaken to find low alkaloid forms for agricultural use (256). No sweet forms were found even though 220,000 plants were examined, and an x-ray treatment was used to produce sweet mutants. Other workers have employed γ-radiation to obtain fodder types (257). Five sweet mutants of *L. luteus* and *L. angustifolius* in a population of 1.5 million plants were found (256). Reference 258 reviews the breeding methods used in the development of low alkaloid lupine varieties.

The inheritance of the sweet and bitter characteristics of lupine species has been studied. There is no doubt that the accumulation of the quinolizidine alkaloids is under genetic control. Hybrids between two sweet varieties may show no change in the amounts of alkaloids accumulated, or (as frequently happens) the hybrid may regain the ability to accumulate large amounts of alkaloids (259, 260, 261). Rarely does the hybrid accumulate less alkaloid than

its parents (260). Crosses of sweet and bitter varieties result in bitter hybrids (254). Genetic evidence has been used in support of a metabolic pathway from sparteine through lupanine and hydroxylupanine to hydroxylupanine ester alkaloids (244, 253). Some species may lack the genes required to form the enzymes necessary to carry out certain of these oxidations (253, 261). Hybridization may result in the reassembly of the genes for the entire pathway (261). Mironenko (259, 261) believes that some fodder varieties completely lack the genes required to form the alkaloids, but other evidence indicates that the fodder varieties can synthesize and utilize the alkaloids but do not accumulate large amounts of them (244, 254). Bitter types seem to lack the genes for low alkaloid content (254). One worker feels that certain genes affect the lupinine/sparteine ratio. The genes for the sweet character increase this ratio; as sparteine is more readily detected than lupinine there is an apparent decrease in the alkaloid content (254).

(4). Ontogenesis of the Quinolizidine Alkaloids in Lupines

Dawson (25) in 1948 discussed the desirability of expressing alkaloid contents of tissues on a per plant or a per organ basis rather than on the basis of the weight of the plant or organ. Studies of the changes in alkaloid

levels during growth and development must be on a per plant or per organ basis to be meaningful. The dry weight of a plant is determined mainly by such cell wall materials as cellulose and lignin and by the protoplasmic protein. Such materials often slowly accumulate as the tissue ages. It is possible that the *absolute* amount of an alkaloid could be the same in both an old and a young leaf, for example; but the older leaf, having a larger amount of cell wall materials *etc.*, would *appear* to have less alkaloid than the young leaf on a dry weight basis. The wet weight of a plant tissue is even less related to the alkaloid content as the water content of tissues is subject to great variations. All of the data discussed below are on a per plant basis. It is unfortunate that all studies of alkaloid ontogenesis in lupines have been reported in terms of weight of alkaloid present (in mg. or μg.) with the exception of the work of Graham and Spencer (161) who report *millimoles* of alkaloid per plant. Precursor-product relationships would become clearer if the reports were made on such a basis (millimoles per plant) that is independent of the variations in molecular weight among the alkaloids. For example, one milligram of sparteine represents one third fewer millimoles than does one milligram of lupinine.

There have been a large number of studies of the changes in the alkaloid content and spectrum of lupines during growth and development. The early development of Lupinus luteus var. Romulus has been divided into stages by Graham (7). He found that the root tip became visible one day after imbibition of water and found the cotyledons green and open after 6.8 days. There were two pairs of leaves present after 14.4 days. Van der Kuy (240) and Wiewiorowski and his coworkers (243, 262) have divided the later growth of lupines into stages. Stage I has two pairs of leaves open, II has ten pairs of leaves, flower buds are formed at stage III and the main stem flowers at stage IV. In L. angustifolius these stages occur at ages of 14, 37, 51 and 67 days respectively. The exact age at which a plant reaches a certain stage varies with the conditions of culture. Often only ages in days are given in the literature. In such cases an approximation of the stage of development is given.

Most workers have found that there is a general increase in the total amount of alkaloids in lupine plants from germination through to flowering and seed pod formation and maturation (234, 253, 249, 262). After seed pod formation the rate of alkaloid accumulation becomes very

low (263, 264, 265). The rate of alkaloid synthesis is low in older vegetative tissues and in lignified tissues (243, 265, 266).

Although the majority of studies on lupine alkaloid ontogenesis have dealt with the growth stages after the primary and secondary true leaves have developed, there is some information available on the alkaloid levels during germination. Alkaloids have not been found present in the seed coat. No changes have been observed in the alkaloid content of dry (albus) seeds during storage, but changes in the types of alkaloids present have been noted after 5 to 6 months storage (264). Of possible interest in this respect is the report that low temperature treatment of white lupine seeds that were incompletely vernalized produces marked variation in the alkaloid content and in the appearance of the resulting plant (268). During the imbibition of water by L. albus seeds Birecka and Wajcieska (264) observed a marked decrease in the alkaloid content of the seeds in some cases. A decrease in the alkaloid content during the earliest stages of germination was also noted by Graham (7) and by Mironenko (269). In sterile culture, lupine plants lost alkaloids in the early stages of growth, but later took alkaloids up from the medium (247). A similar loss of alkaloids from germinating seeds was noted by Fontanelli (270).

The alkaoid content of sterile cultures of *Lupinus angustifolius* cotyledons dropped from the onset of germination to about the sixth day *without* a concommitant increase in the alkaloid content of the medium (246). It should be noted that the alkaloid levels of plants in tissue culture differed quantitatively, but not qualitatively from plants grown normally. Reifer has shown that alkaloids are synthesized (and metabolized) in the cotyledons *only* during the first four days; after the fourth day removal of the cotyledons did not affect the synthesis of alkaloids by the plant (246). This was confirmed by a demonstration that cultured blue lupine embryos did not form alkaloids until after the fourth day. Other workers have reported no change in the total alkaloid content during nine days (to primary leaf opening stage) after germination of *L. albus* (264). The alkaloid content drops in etiolated seedling (235). Graham (7) found that lupinine increased during the first five days (cotyledons green; primary leaves beginning to develop), then decreased and began to rise again with the opening of the first leaves. Mironenko (269) observed that the alkaloid content decreased early in the development of yellow lupines, but that the alkaloid level has begun to increase at the time of the first leaf opening. He noted that lupinine rose more rapidly than did sparteine. Graham (7) observed an initial decrease, and

then disappearance of tetracyclic quinolizidine alkaloids (probably sparteine) accompanied by a marked rise in the lupinine level. Tetracyclic alkaloids reappeared after seven to nine days (as primary pair of leaves opened) and slowly increased. The decrease in alkaloid content during early germination is of interest. It could indicate conversions among the alkaloids (e.g. tetracyclic alkaloids ⟶ lupinine) or it could indicate conversion of the alkaloids to non-alkaloid materials. The decrease in lupinine levels after 4 to 5 days found by Graham (7) is not accompanied by any marked rise in tetracyclic alkaloids and might be interpreted as conversion of lupinine to non-alkaloid materials.

During later stages of growth both the total alkaloid content and the levels of the individual alkaloids rise quite uniformly up to 40 to 65 days (beginning of flower bud formation to flowering) (240, 265). During flowering there is often a very dramatic rise in the rate of alkaloid accumulation (234, 243, 262, 263, 264, 265, 266). A rate of accumulation of about 16 mg. of alkaloid per plant per day has been noted at this stage (243). After flowering and seed pod maturation the rate of alkaloid accumulation drops markedly (265, 266). The flowering of the side shoots (begins at about 85 days) contributes a large increase in the

rate of alkaloid formation (264, 265). At this time the central stalk is virtually quiescent and the side shoots contribute 70 to 90% of the total alkaloid accumulated by the plant (265). Alkaloids are particularly concentrated in the developing seeds, but the seed pod does not itself synthesize or accumulate significant amounts of quinolizidine alkaloids (265, 266). The observation that there are significant differences in the characters as well as in the quantities of alkaloids accumulated by fodder and bitter varieties (266) is worthy of note.

Changes in the levels of the individual alkaloids during later development have been observed. In *L. luteus* lupinine levels remained nearly constant during the first 40 days (up to start of flower bud formation) and then fell to zero. Sparteine and lupanine slowly increased with a marked rise at 55 (late flower bud formation) and 65 (early main stem flowering) days, respectively (240). Lupinine reappeared and rose rapidly and continuously after 65 days. Sparteine dropped and then increased between 80 and 90 days (start of side shoot flowering) while lupanine levels began to fall (240). In *L. mutabilis* lupinine rose to a peak at 40 days (start of flower bud formation) then fell only to increase again at the onset of flowering after which it showed little change. Sparteine and lupanine rose throughout the growth

of this species. *L. albus* showed a drop in lupinine accompanied by an increase in sparteine up to 40 days. Sparteine and lupinine then uniformly increased (240) although in one year sparteine levels dropped towards the end of the plant's life cycle. Other workers observed that sparteine decreased while lupanine and hydroxylupanine increased during flower bud formation in the white lupine (248, 271).

Lupinus angustifolius has been the subject of some particularly interesting studies of alkaloid ontogenesis. In 1962 a decrease in the alkaloid content was observed to occur during the first growth stages followed by intense biosynthesis up to the time of flowering (263). A similar pattern was reported in 1963 with a minimum in the levels of the total alkaloids, and in lupanine, hydroxylupanine, and angustifoline levels. After four days (cotyledons green) the total alkaloid content then increased. At ten days of age (primary leaves open, secondary leaves beginning) a fraction consisting of hydroxylupanine esters appeared and rose rapidly (246). This was re-investigated by a large group of workers in Poland. They found that the total alkaloid content rose after 14 days (secondary pair of leaves open) as did the content of 'normal' (not esterified) alkaloids. The level of hydroxylupanine ester alkaloids rose

until flower bud formation after which it fell to low levels. The alkaloids lupanine, hydroxylupanine and angustifoline rose during growth. The hydroxylupanine esters increased very rapidly and reached a peak when the plant had developed 10 pairs of leaves after which the rate of accumulation decreased slightly. After 50 days (flower bud formation) lupaninic (lupanic) acid appeared and its level rose rapidly (243, 262). On the basis of these ontogenetic studies these workers have proposed a new scheme for the biosynthesis of some of the tetracyclic quinolizidine alkaloids. This scheme will be further discussed in a later section.

The methods used to isolate the hydroxylupanine ester fraction have been varied (160, 165, 243, 251), however the possibility of esterification during isolation has not been completely eliminated. It is possible that the appearance and changes in the levels of these ester alkaloids might occur because of organic acids or non-alkaloid esters extracted from the tissue which react with the hydroxylupanine during isolation. Aqueous extraction and very gentle isolation and separation methods should be applied to show the presence of these ester alkaloids. The lupaninic acid was shown not to be the result of lupanine hydrolysis during isolation (243).

Ontogenetic studies of quinolizidine alkaloids in the genus Cytisus have also been published (272, 273). Increases and decreases in alkaloids levels in Cytisus indicate that the quinolizidine alkaloids may be intermediates in the metabolic activities of the green parts of the plant. Alkaloid levels were highest in the seeds, fruits and flowers. Lupanine was found to increase as sparteine decreased.

With the exception of dry and wet weights of the plants, there are no studies relating alkaloid ontogenesis to the overall metabolic activity of the plant. It would be of interest to see if the periods of rapid alkaloid accumulation or degradation coincided with periods of high general or specific metabolic activity. Such parameters as respiratory and photosynthetic rates, free amino acid metabolism, and protein synthesis might be compared with alkaloid metabolism.

(5). The Site of Quinolizidine Alkaloid Formation in Lupine Plants

It is often difficult to distinguish between the site of alkaloid accumulation and the actual site of synthesis in the plant. Studies based upon the feeding of known, labelled alkaloid precursors to isolated parts of the plant are most useful. Such studies, in addition to grafting

experiments, have shown that the leafy, green parts of the plant carry on most of the alkaloid formation.

The ability of the root to form alkaloids and to accumulate them has been debated. There have been some reports of alkaloid formation in root tissues (22, 25, 274). Mothes and Kretschmer found that the alkaloid content of *Lupinus angustifolius* roots in sterile culture increased proportionally to root growth (275). Grafting experiments have given some evidence for alkaloid formation in the root (274). Roots are able to accumulate alkaloids, but the amounts are generally low. Some workers report finding alkaloids in the root (276) while others report that there is little or none present (26, 243, 277). Alkaloids have been shown to be translocated to the root (244) and are leached from the roots into the soil (278). Most grafting experiments have shown that a sweet scion on a bitter stock does not accumulate the amounts or kinds of the alkaloids characteristic of the bitter stock (274, 279, 280, 281). The alkaloids of a bitter scion on a sweet root stock are the same as those in a control bitter plant. Other studies have shown that derooted plants can carry on normal alkaloid synthesis, so the roots are not essential even for the formation of alkaloid precursors (282). (The roots may form some, but there are apparently other sources in the plant as

well.) Relative levels of the different alkaloids are altered in derooted plants, however, so it is possible that the root has some sort of indirect control over alkaloid synthesis and degradation.

Grafting experiments have shown that only the green parts of the plant have any major role in alkaloid synthesis (253, 274, 279). Alkaloids from a bitter scion can be translocated to other shoots on a sweet root stalk (244, 279). Derooted plants of yellow and white lupines can accumulate alkaloids at the same rates as intact control plants (282, 283, 284). Isolated leaves can incorporate $^{14}CO_2$ into alkaloids (282). Some workers have reported alkaloids levels to be low in lignified (i.e. older) stems (243). Others have found higher alkaloid accumulations in stem tissues (26, 276). The stems of plants exposed to $^{14}CO_2$ were found to contain alkaloids of far greater specific activity than that of the alkaloids in the leaves (282). Stems of derooted plants were found to increase more in weight, protein content, and quinolizidine alkaloid content than did the leaves (283). In lupine cuttings, the petiole was observed to be more active in alkaloid synthesis and interconversions than either the leaves or the stems (284). It can be safely concluded that the green parts of the plant carry on most of the biosynthesis of the quinolizidine alkaloids, although the roots may have a

low level of biosynthetic activity. Whether the leaves or the stems are most important to alkaloid synthesis is not clear as most of the experiments performed cannot properly distinguish between synthesis in the stem and translocation to it. It has been shown that leaflets alone have the ability to synthesize quinolizidine alkaloids. Alkaloids are also present in flower tissue (276) whether by translocation or by synthesis is not clear.

The relationship between alkaloid biosynthesis and the photosynthetic activity of the green parts of the plant is of interest. White and Spencer (26) found that alkaloids were most densely concentrated in the cotyledon tissues most active in photosynthesis. Dark grown seedlings had little or no alkaloid in these tissues. Alkaloids are known to be lost from etiolated lupine seedlings (235). Some workers have reported that alkaloid levels do not rise in germinating seedlings until the fourth day (246, 247). At this time the seedlings are becoming green (7). Whether the seedling actually synthesizes alkaloids before the fourth day has not been demonstrated clearly as during this period the total alkaloid level per plant drops (246, 247). It is possible that the overall drop (perhaps in the alkaloids stored in the seed) may cover some synthetic activity. Tracer studies are needed to examine this possibility. Birecka and

Zebrowska (239) observed diurnal changes in the alkaloid levels of L. albus and L. luteus. Alkaloid levels in L. albus rose during the day and fell at night. Most of the changes occurred in the alkaloids of the leaf blades. During the day the alkaloid levels of the yellow lupine either rose or stayed constant and showed no change during the night. Sparteine in the leaves decreased during the day and increased during the night while lupinine levels rose during the day and fell at night. If lupinine is a precursor of sparteine (as seems probable – lupinine has been shown to be converted to sparteine, but the reverse does not occur), this might indicate that the initial alkaloid formation was light-dependent, but that the later reactions were not. Birecka (285) found that in young white lupine plants there was little incorporation of photosynthetically fixed carbon dioxide into alkaloids and that cotyledons were inactive in this regard. Plants that had maturing seed pods showed much higher incorporation of $^{14}CO_2$ into quinolizidine alkaloids than did younger plants. At this stage there is intense translocation of organic materials into the seeds. Further studies on the involvement of alkaloid formation and photosynthetic activity are needed.

(6). Some Physiological Aspects of Quinolizidine Alkaloid Formation

Alkaloid formation is known to be most active in actively growing tissues and decreases as the tissues age (265, 266).

Studies on the effect of certain nutritional elements on the accumulation of alkaloids in lupines have been carried out in Russia and Eastern Europe. All of the studies have been agricultural in nature, but the results are of interest to the consideration of the place of the quinolizidine alkaloids in plant metabolism. Hydroponic and tissue culture nutritional studies should be performed.

Phosphorus increases the alkaloid content of lupines after a certain optimum phosphorus level is reached (286, 287, 288). Alkaloid formation and nitrogen assimilation are decreased in phosphorus deficiency but fat accumulation was unaffected (288). Phosphorus in the presence of potassium and boron increased the yield of lupines, but decreased the alkaloid content (289). Alkaloid content was found to be lowest when the ratio of potassium to phosphorus was highest (290).

Nitrogen fertilizers increased the alkaloid content except when low levels were used on nitrogen-deficient soils (289, 291).

Potassium raises the yield and growth rate of lupines without increasing the alkaloid content (289, 290, 291). Lupines can release potassium that is strongly bound to certain clays (292). Potassium appears to counteract the alkaloid increasing effect of phosphorus (289, 290).

Boron decreases the alkaloid content of lupine plants while increasing growth, protein synthesis, potassium uptake, and nitrogen content (293, 294, 295). Molybdenum also decreases the alkaloid content while increasing growth and protein synthesis (293, 295). Manganese has no discernible effect on lupine plants (293, 295). Copper either has no effect (239) or is deleterious to the plant's growth (295).

Kinetin treatment produces a two-fold increase in the alkaloid content of cultured L. luteus embryos, while adenine, β -indole acetic acid, and kinetin plus β -indole acetic acid produce very large increases (up to 170%) in the alkaloid content (296). Gibberellin A 3 had no lasting effect on lupine growth and did not affect the alkaloid or nitrogen contents of the plants (297). The nitrogen content of yellow lupines treated with β -indole acetic acid increased as did the yield. The alkaloid content of yellow lupines was increased by α -indole acetic acid spraying while the total nitrogen content was not altered (297).

(7). Amino Acid and Protein Metabolism of Lupine Plants

The basic amino acid lysine is commonly accepted as the precursor of the quinolizidine alkaloids in lupines (250, 298). The levels of lysine (both free and protein-bound) in fodder and bitter varieties of lupines are therefore of interest.

The total nitrogen content of *Lupinus luteus* plants was found to be 32.15%; the ratios of nitrogen to sulphur and of nitrogen to phosphorus were 11.8 and 7.4 (299). No differences in the total nitrogen content and the non-protein nitrogen content were found between fodder and bitter types of lupines (300) (this reference does not state whether alkaloids are included in the non-protein nitrogen figures, but it is presumed that they are not, as alkaloids are frequently precipitated by the common protein precipitants). *L. angustifolius* proteins are high in cystine and lysine and are low in arginine and histidine, while *L. albus* proteins are high in arginine, lysine, and cystine (299). *L. luteus* proteins are low in lysine and cystine and are high in arginine and histidine (299). The three species are listed in order of their relative alkaloid content. Bitter types of lupines are low in lysine, glutamic acid and arginine compared to sweet types, and these levels remain constant during development as do the levels of these

amino acids when free (301, 302). Free amino acid levels are possibly more directly related to alkaloid biosynthesis. Levels of free lysine, arginine and glutamic acid are higher in sweet lupines than in bitter (302, 303). Some workers have found arginine to be higher than lysine in certain sweet varieties (130) and others have found free lysine to be high in bitter types (301). Histidine and phenylalanine are higher in bitter lupine varieties than in sweet, while arginine, glutamic acid and threonine have been found to be the reverse (304). Free amino acid levels have been found to be high in older roots and in immature seeds (301). Immature seeds have only traces of free amino acids. More recent work has shown lysine to be the main basic free amino acid of both sweet and bitter yellow lupines, although the free basic amino acids constituted just 1 to 3% of the total basic amino acids (298). Lysine levels in organs active in alkaloid formation do not differ from those of inactive parts of the plant (298). Graham (7) has studied the ontogenesis of the basic amino acids during the first twenty days of growth. He found that free lysine was higher than free arginine and histidine; the latter two showing no marked changes with ageing. Lysine, however, rose sharply on germination, then fell and rose again, behavior similar

to that of lupinine. The reverse behavior might have been expected as the lysine content should decrease as it is converted to the alkaloid.

(8). Proposed Functions for the Quinolizidine Alkaloids

It has been suggested that the quinolizidine alkaloids protect the lupine plants that accumulate them from attack by aphids (305). There was a negative correlation found between alkaloid content and aphid infestation in a randomized field experiment. Nowacki (306) did not find quinolizidine alkaloids protected plants from insects or molloscs, and Smith (307) reports an aphid that will only feed if sparteine is present! Nowacki was also unable to find any effects of alkaloids on growth and development and on the attack of microörganisms (306). Alkaloids are known to leach into the soil during germination (270) and from roots (278). A suggestion has been made that alkaloids exchange against soil cations, thus having a 'metal-sparing' action (278). Quinolizidine alkaloids are known to inhibit the germination of sweet lupine seeds (308). In the soil quinolizidine alkaloids are known to be decomposed by microörganisms (235).

Sparteine inhibits invertase (309) and monoamine oxidase (310) *in vitro*. Sparteine also activates some metalloenzymes isolated from sugar beets (311). Lupinine and

some tetracyclic alkaloids in the presence of Fe(III) have distinct catalytic behavior in some reactions (for example in H_2O_2 decomposition) (312). In _Senecio_ there is evidence for oxidation and reduction of the alkaloids and alkaloid N-oxides (324). As N-oxides occur in the company of reduced quinolizidine alkaloids (46) it is possible that these alkaloids also may have a role in some redox reactions. The dynamic changes observed in the alkaloid content during periods of active growth argue for an active part for the quinolizidine alkaloids in plant metabolism (7, 161, 313).

(9). Other Studies with Lupine Plants

Lupine plants have been used in physiological and biochemical studies unrelated to their alkaloids. The softening of the seeds (314) and osmotic responses of the seedling (315) have been investigated. The abscission of lupine leaves has been studied (316, 317) and the abscission promoting factor dormin (abscisin II) has been isolated from lupines (318, 319) and its interactions with other growth hormones studied (320). The gibberellins of lupines have also been studied (321). Lupine plants have been compared to sunflowers and beans with respect to their ability to translocate products of photosynthesis (322). Some of the properties of isolated lupine mitochondria have been studied and their tricarboxylic acid cycle activity examined (323).

E. AMINE OXIDASES AND THEIR POSSIBLE ROLE IN QUINOLIZIDINE ALKALOID BIOGENESIS

(1). Introduction, Definitions, Nomenclature, Reactions

Diamine oxidase is thought to be involved in the conversion of cadaverine (or lysine) to the precursors of the quinolizidine alkaloids. This review of the literature on the amine oxidases is more extensive than might be considered necessary for a thesis. However, an extensive review was necessary, as a recent review was not available, nor was there any review on the amine oxidases of plants and their possible role in alkaloid biosynthesis. Since the preparation of this literature review two related reviews have been published. That of Buffoni (325) is a particularly good summary of animal amine oxidases with emphasis on their role in mammals. Reference 326 purports to be a review of "some recent advances in the field of amine oxidases", but is really a discussion of beef liver mitochondrial amine oxidase only. No review on the plant amine oxidases has yet been published.

Amines are ubiquitous in living things and their metabolic roles, where known, are widely diversified. Such amines as histamine and its relatives are of importance in the nervous system and have an apparent hormone action on the organism as a whole (325, 327, 328). Diamines and polyamino

compounds are known to interact with polyanions such as the nucleic acids. Diamines influence membrane stabilities, may serve as growth factors, bacteriacides, and have been implicated, in both plants and animals, as the precursors of certain heterocyclic compounds (329, 330, 331). It has been speculated that the indole auxins may be derived from amine precursors (332). A group of enzymes known as amine oxidases has been shown to carry out the oxidative deamination of various amino-group-containing compounds (325, 329, 333). The generalized equation for such enzymes may be written as:

$$R\text{-}CH_2NH_2 + O_2 + H_2O \rightleftharpoons RCHO + NH_3 + H_2O_2$$

In the presence of catalase the stoichiometry becomes:

$$2RCH_2NH_2 + O_2 \rightleftharpoons 2RCHO + 2NH_3$$

The resultant aldehyde, depending upon the nature of the rest of the molecule, may undergo further spontaneous reactions, as in the case of certain amino aldehydes that spontaneously undergo a Mannich-type condensation to form N-heterocyclic compounds of possible importance in alkaloid biosynthesis.

It would appear that a large group of enzymes, more or less closely related, is capable of oxidatively deaminating amines. These enzymes are often flexible in their substrate specificities *i.e.* an enzyme capable of deaminating a certain amine most rapidly may also be capable of acting upon the substrate characteristic of another amine oxidase. Much of the

earlier naming was done on the basis of the particular substrate used in the assay, or the substrate found to be the most rapidly oxidized under the experimental conditions employed, or the substrate that was perhaps just the most convenient. The literature thus contains considerable confusion over the identities of these enzymes. It is now generally agreed that those enzymes that are most active towards diamines and that are inhibited by carbonyl reagents (such as semicarbazide) are known as 'diamine oxidases' ('DAO') (329, 334). These enzymes are also known as semicarbazide-sensitive amine oxidases (329). Enzymes that preferentially attack monamines and that are not sensitive to carbonyl reagents are known as 'monoamine oxidases' ('MAO') (329, 335). It has been demonstrated that monoamines may act as substrates for DAOs (335, 336, 337). Similarly, certain diamines are substrates for MAOs (333). For this reason some prefer to refer to both of these enzyme groups as 'amine oxidases' (6). Although this usage perhaps removes the ambiguities in the substrate specificities it does not differentiate between these two groups, which do differ markedly in their inhibition characteristics, in their intracellular location (329), and, perhaps, in their cofactors. To further confuse the issue, Gorkin and Tatyanenko (338) have recently reported the conversion of a monoamine oxidase to a diamine oxidase-like

enzyme *in vitro*. However the treatments used to produce this interconversion involved heating or treatment with fatty acid peroxides, of a monoamine oxidase from rat liver mitochondria. For the purposes of this review, it is understood that diamine oxidases are enzymes that attack diamines preferentially and that are inhibited by carbonyl reagents, but not by sulphydryl group inhibitors. Monoamine oxidases are insensitive to carbonyl group inhibitors, are sensitive to sulphydryl inhibitors, and show preference towards monoamine substrates.

Histamine, with its nitrogen-containing ring, may be degraded by DAOs (339). This property has lead to debate on whether the histamine destroying enzyme (histaminase) is identical with diamine oxidase. Studies (mainly done by Zeller and his coworkers) on purified animal DAO seem to indicate that histaminase is identical with DAO (329, 335, 340, 341, 342, 343). This prevalent theory is disclaimed by Kapeller-Adler who gives evidence that DAO and histaminase activities are separable (344, 345, 346). Evidence supporting this is given by Werle and Hartung (347), Blaschko and Hawkins (348), and by Goryachenkova (349). Zeller argues that the microvolumetric titration used by Kapeller-Adler (350) to determine the hydrogen peroxide formed during the reaction, is not a well defined stoichiometric method (329, 335) and others have pointed out possible sources of error in

the reaction conditions used for the measurement of the substrate specificity (334). Kapeller-Adler's work should not be so glibly discounted and considerable light could be shed on the question if other activity assay methods were to be used. Smith (351) has recently reported the purification of a placental histaminase that maintained a high diamine oxidase activity throughout the purification procedure if EDTA was present in the buffers. In the absense of EDTA histaminase activity remained high, but diamine oxidase activity fell during purification. Resolution of the question as to whether or not histaminase and DAO are different awaits further study. In studies of the plant enzyme both terms have been used interchangeably (336, 337). Moreover the plant enzyme differs from the animal enzyme. The activities towards diamines and towards histamine have very different requirements for optimum activity (e.g. pH) and so direct comparison of the two activities in any one enzyme preparation is difficult (340).

Other enzymes generally classed as DAOs, which may or may not be sufficiently distinct to warrant individual names are: spermine oxidase, Serratia spermidine oxidase, putrescine oxidase (Micrococcus rubens) (352), and plasma amine oxidase (329). Buffoni divides the amine oxidases related to histaminase into four groups: mammalian intracellular

amine oxidases (various histaminases and diamine oxidases - he considers the two types to be separate but related), plasma benzylamine oxidase (which acts only on monoamines but is inhibited by carbonyl reagents), plasma spermine oxidase, and the plant and microbial amine oxidases (325). It is probably incorrect to class all of the microbial amine oxidases together as they differ widely in properties and in cofactor make-up. Some enzymes are similar to the mammalian enzymes while others contain flavin adenine dinucleotide (FAD).

Nara and Yasunobu suggest naming the amine oxidases as follows: source of enzyme, cellular localization of enzyme, followed by the general name - amine oxidase (326). They also suggest classifying the amine oxidases on the basis of the prosthetic groups. Such a method will probably prove very useful when more amine oxidases have been subjected to careful study. At the moment the nomenclature of the amine oxidases is very confused and the properties, rather than the names of the enzymes reported in the literature must be considered. The amine oxidase isolated from plants, despite its wide substrate range, is sensitive to carbonyl reagents and insensitive to sulphydryl group reagents and has most of the other properties of a diamine oxidase (337) and as such it will be considered. The enzyme is officially classed as diamine-oxygen oxido-reductase EC 1.4.3.6 (deaminating) (337).

(2). Sources, Distribution and Intracellular Location

Diamine oxidases and related enzymes are widespread among living things. They are found in micro-organisms (329, 339, 353, 354), including *Escherichia*, *Pasteurella*, *Pseudomonas*, and *Streptococcus*. *Serratia marcescens* forms a particularly specific spermidine oxidase which will not attack spermine (329). Diamine oxidases are present in higher plants (336, 339, 341, 355, 356). DAO has been demonstrated in legumes (including pea, bean, clover and lupine) (336, 341, 357, 358), *Lavandula* (341), *Atropa* and *Datura* (356), and in hemlock (355). It is absent in some plant families (341) and is not detectable in resting seeds as for example the case of *Lupinus* (359).

There are few, if any, references to DAOs in lower plants, although amine oxidases have been shown to play a role in the metabolic activities of some fungi when grown on diamines as the sole carbon source (360, 361, 362).

The DAO activities of vertebrate tissues show marked variations (some of which are possibly artefacts). The enzymes from different sources (*e.g.* kidney and plasma) from the same animal may show differences in behavior, especially in substrate specificity (363). High enzyme levels are found in tissues participating in transport of large amounts of material (329). Kidney is high in DAO and

is a common source (334, 364, 365). Liver, lung, skin, certain glands (329), intestine, and placenta (366) show DAO activities as does human blood plasma during pregnancy (334, 367). The activities in brain tissue (in which MAOs important) seem to be variable - some have demonstrated its presence (366, 368), and others its absence (329). However, recent tracer evidence (329) suggests that, with the exception of carp brain, there seem to be no DAOs in brain tissues. The possibility exists that technical differences may have caused these discrepancies or that the presence of the enzyme may be species specific as is that of plant DAO.

Diamine oxidase in mammals may have several functions. Its action on histamine is, of course, important in the functioning of nerve tissue. Its increase in plasma during pregnancy is unexplained, but the anticoagulant heparin has been observed to cause a similar increase in plasma diamine oxidase (369). Perhaps diamine oxidase has some role in blood clotting or its associated processes? Diamine oxidases seem to be involved in the conversion of lysine residues to cross linkages in elastin and tropocollagen (370, 371). Elastin synthesis is deranged in cases of deficiency of copper or pyridoxal phosphate, both constituents of DAO. Diamine oxidase is also high in blister fluid (372), as well as reducing shock reactions in guinea pigs (373).

Diamine oxidase is formed during seed germination in plants. There is no DAO activity found in ungerminated seeds, nor is there any in freshly soaked pea seeds. Activity first appears after 3 to 4 days, is maximal at 7 to 18 days and decreases as the cotyledons age (336). The mature pea plant at harvest shows some DAO activity and occasionally there is activity in the root nodules (336). Later studies show that amine oxidase appears 24 hours after imbibition of water (359). DAO has been shown to be present in the hypocotyls of lupine seedlings (374) and is formed in the cotyledons of peas after removal of the embryo (359). Substrates for DAO have been found in the seedling root and shoot, but not in the dry seeds. Histamine is present in the germinating seeds of many plants and its formation seems to be linked to cell multiplication (375). It has been reported that DAO inhibitors retard the lengthening of lupine roots and that pyridoxal phosphate stimulated lengthening (376). These observations give indication that DAO may be involved in growth or growth regulation. Suzuki has shown that there is no lysine decarboxylase activity in lupine seedlings, but a DAO is present - he gives evidence for the formation of tetrahydroanabasine in the hypocotyl (374). It was also shown that lysine oxidation in the lupine seedling occurred via DAO, not by a transamination or

by a peroxidase coupled reaction (377). The most convenient sources of plant DAO are legume seedlings - pea, clover, lupine, etc. The first two have seen particularly extensive use (337, 347, 378).

Diamine oxidases are generally present in the 'soluble cytoplasmic phase' of the cell (325, 329). Although no thorough studies have been made of this with the plant enzyme, the extraction and purification procedures used are such that particle-bound activity would be lost (337, 378). The mescaline oxidase from rabbit liver is an exception to the generally assumed localization of DAOs, as it is found in the microsomal and other particulate fractions (329). Cotzias has reported a DAO associated with particles from liver (357), but does not report any inhibition data to demonstrate that the histamine degradation he obtained was indeed caused by a typical DAO and not by a MAO.

Monoamine oxidases are found bound to the particulate fractions of tissues (generally the mitochondrial fraction) (325, 379, 380, 381, 382) and often present solubilization difficulties requiring detergent treatments and/or sonication for their rectification (383, 384, 385, 386). Monoamine oxidase is present only in the outer membrane of rat liver mitochondria and is suggested as an enzymic marker for the

outer membrane (385). MAO activities have also been demonstrated in rat liver microsomes (333).

Monoamine oxidases have been shown to be present in such plants as Colchicum, Cannabis, and Salvia (386, 387). This enzyme acts in the presence of DAO inhibitors and shows preference for monoamines over diamines. A monoamine oxidase inhibitor has been shown to inhibit the germination of Raphanus seeds (388).

(3). Extraction, Purification and General Properties of Plant Diamine Oxidase

The development of the purification procedure discussed below is of interest as it is the procedure I have used to prepare DAO for studies of its role in quinolizidine alkaloid biosynthesis. Kenten and Mann (336) prepared a crude diamine oxidase from pea seedlings by grinding them with water, followed by an ammonium sulphate precipitation, dialysis, and precipitation with acetone. The dry acetone powder was relatively stable at room temperature (lost 60% activity in a month). Mann later modified this procedure by using phosphate buffer to extract the enzyme, followed by treatment with cold ethanol-chloroform, an ammonium sulphate precipitation (which left peroxidase activity in the supernatant) and finally repeated precipitations by adjustment of the pH from 7 to 5. The enzyme preparation was very stable

at -10°C (389). Further purification was achieved by Mann (390) who followed the above procedure by chromatography on amino-ethyl- or diethyl-amino-ethyl-cellulose columns then by chromatography on a hydroxyapatite column to obtain a rose-red enzyme solution that was very stable when frozen and that represented an 880-fold purification. Hill and Mann (1964) (337) added an 18,000 g centrifugation step after the pH 5 precipitations - the purification obtained is not stated but is probably similar to that from the 1961 method. Werle and his co-workers used a similar procedure with kieselghur column chromatography to obtain a rose-red preparation representing a purification of 1300 fold (329, 378). Diamine oxidase from yellow lupine seedlings has recently been purified by ammonium sulphate fractionation and by column chromatography on diethyl-amino-ethyl cellulose, celite, and hydroxyapatite (391). This procedure achieved only a 250 fold purification, and the colour of the final product is not mentioned although the impure preparations were brown. Uspenskaia and Goryachenkova (392) employed zone electrophoresis to purify pea seedling DAO. They found, as was not found with the DAO from hog kidney, that separation by electrophoresis was not very effective, as the inactive contaminating proteins had similar electrophoretic mobilities to the enzyme under the experimental conditions. Furthermore, they found the

enzyme extremely labile when free of the inactive proteins. They obtained a purification of 545 fold. There has been no report published on the crystallization of plant DAO, nor has there been a purification of greater than 1300 fold.

Similar applications of standard enzymological techniques have been used to prepare DAOs from other sources (329, 364, 365, 366). Some crystalline preparations have been obtained (329, 393). Pig kidney diamine oxidase has been purified 3,250 fold by procedures that include column chromatography and electrophoresis (394, 395). This preparation was "pink-yellow" and was homogeneous by gel electrophoresis and ultracentrifugation.

Preparations of plant DAO are quite stable-thermally and chemically. The standard purification technique, as developed by Mann, involves treatment with cold ethanol-chloroform, but many of the subsequent steps in the procedure could be performed at room temperature (378, 37 9). Acetone treatments have also been used in amine oxidase preparations (336, 348). The crude acetone powder prepared by Kenten and Mann lost 30% of its activity when stored at room temperature for two weeks (336). More highly purified preparations are very stable when stored cold (337, 389). A 300 fold-purified preparation from pea showed little or no loss of activity when stored frozen in solution (-10°) for several months

(389, 390). It is of interest to note, however, that Goryachenkova and Uspenskaia reported that the DAO they purified from pea extracts by starch gel electrophoresis was very unstable once removed from the contaminating proteins and extracted from the starch matrix (392). The 1300-fold purified DAO of Werle *et al.* was not as stable at -10° as were less pure preparations. They found that lyophilization improved the storage properties (378). Repeated freezing and thawing in the presence of certain DAO inhibitors has been shown to release pyridoxal phosphate from purified pig kidney DAO (394). Kapeller-Adler reported a high degree of stability at 4° and at -20° for her histaminase preparations (346). Purified hog kidney DAO was found to lose 80% of its activity in 20 days at 0 to 4° and 50% of its activity after two months frozen at -20° (395). Stability was higher in very concentrated solutions (*i.e.* 40-50 mg/ml). Lyophilization gave a powder which was stable without appreciable loss of activity for several months if kept cold and dry. Incubation of pea DAO with trypsin or chymotrypsin for two hours at pH 7 did not affect DAO activity (378). This is in contrast to the behavior of crude hog kigney DAO towards trypsin treatment (329).

Purified hog kidney DAO precipitates (without denaturation) at neutral pH and low ionic strength (394).

Electrophoretic studies by Kapeller-Adler and MacFarlane on pig kidney histaminase indicated an isoelectric point between 5.00 and 5.15 (345, 346). Pea DAO may be precipitated by adjustment of the pH from 7 to 5, which indicates a similar isoelectric point to that of histaminase. Uspenskaia and Goryachenkova found that the electrophoretic mobility of pea DAO was -0.7×10^{-5} cm^2/sec./volt in 0.05M phosphate buffer (pH 7.2) at zero degrees (392).

Purified pea DAO is stable for at least two hours between pH 3 and pH 10 at 37° (378). The pH optima for DAOs are quite variable and depend upon the particular enzyme source and upon the substrate used. Werle _et al._ reported optima for pea DAO of pH 7.0, 7.5 and 8.0, for cadaverine and histamine, tyramine, and β-phenylethylamine respectively (378). Kenten and Mann found their pea DAO preparation gave optima of pH 7.5 for putrescine, 6.0 for cadaverine, and 8.0 for hexamethylenediamine (336). The formation of indole from 2-(2-aminophenyl) ethylamine catalyzed by pea DAO had its pH optimum in the range 8.8-8.9 (332). The pH optimum for lupine DAO is between 6.9 and 7.1 (391). It is of interest that a model reaction proposed for amine oxidation (see below) procedes best at slightly alkaline pH values (396).

The most common buffers for diamine oxidase studies are those based on (_ortho_) phosphate (326, 329, 341, 390, 394)

and 0.067M is frequently used (336, 378, 390, 397), as is 0.1M (341), usually at pH's of 6.5 to 8.5 (336, 389, 390, 397). Mann and Smithies used (*ortho*)-phosphate-borate buffers at pH 8.5 with good results in their study of indole formation (332). Usually potassium salts are used, although in some cases the sodium phosphates are used as well (390). Mann found that the slow secondary oxygen uptake that occurred after the rapid uptake of the stoichiometric oxygen requirement, which was probably the result of oxidation of the primary oxidation products from certain substrates (*e.g.* cadaverine), catalyzed by traces of metals and/or catalase, could be inhibited by the use of *pyro*-phosphate in place of *ortho*-phosphate buffers (331, 389). Tris buffers have also been successfully used (398).

There is very little molecular weight data available for plant DAO. The only figure is that computed by Hill and Mann on the basis of electron microscope and ultracentrifuge data for a pea DAO preparation (better than 800 fold purified - showed less than 5% of faster sedimenting materials). They estimated the molecular weight to be about 96,000 (337). In the same paper they report the copper content to be one gram atom per 73,000 grams of enzyme which (in the case of one gram atom copper to one mole of enzyme) would tend to confirm the 96,000 molecular weight figure, as the preparation was not

fully pure and as the copper content determination used did not permit a high degree of accuracy. The assumptions made in the molecular weight computation may also have introduced considerable error. A 250-fold purified lupine DAO preparation was not homogeneous in the ultracentrifuge and had s^{o}_{20} of 7.75 (337) so the 6.0S peak is probably DAO. Crystalline bovine plasma amine oxidase had a molecular weight of 255,400 ± 20,000 (329). This enzyme had a copper content of approximately four gram atoms per mole, *i.e.* one gram atom to 61,000 to 71,000 grams of protein (329). Pig kidney diamine oxidase has been prepared that is homogeneous by gel electrophoresis and in the ultracentrifuge (394, 395). The $s_{20,w}$ value for this preparation was 8.4 to 9.0 and the molecular weight was in the range 119,500 to 135,000. The sedimentation coefficient was found to increase with increasing enzyme concentration. This is the opposite of the behavior expected for the concentration dependence of the sedimentation coefficients for a non-interacting component. The observed behavior is taken as evidence for dissociation at low concentrations. The molecular weight obtained was not a multiple of the minimum molecular weight (87,000) obtained by copper analysis (by atomic absorption). These workers do suggest that the higher molecular weight obtained by sedimentation experiments may be a result of the enzyme being made up of more than one 87,000

molecular weight subunit. They consider that most amine oxidases are built of more than one subunit of molecular weight 80,000 - 100,000, each containing one gram atom of copper (395). More studies should be undertaken to examine the molecular weights of the diamine oxidases and to examine the possibility that they have a subunit-type structure. It appears possible that plant DAO represents the smallest active unit of the enzyme.

Diamine oxidase is precipitated by ammonium sulphate at half-saturation (329, 389).

Highly purified preparations of diamine oxidase are pink to rose-red (378, 390) or are 'yellow-pink' (394).

Various methods have been devised to study of locales of amine oxidases in tissues. Although the majority of histochemical techniques have been developed for MAOs, some work has been reported with DAOs. The forensic applications of DAO tests have seen development as they are convenient means for detecting blood and sperm in trace amounts (399, 400, 401). Cold acetone fixation has been used to reduce the diffusion of DAO in tissue sections (402), and as inhibior based histochemical differentiation of amine oxidases using the Fuelgen reaction has been published (403). There is no record of any studies on the localization of DAO in plant tissues with the exception of its presence in seedling leaves (359) and in the lupine seedling root (376).

(4). Studies on Diamine Oxidases

(a). substrate binding and specificity

A wide variety of substrates may be oxidized by the amine oxidases. Table V gives the names and structures of several of the more frequently employed amine oxidase substrates. Diamine oxidase can also oxidize certain monoamines.

Pig kidney DAO has its highest affinity for diamines with four methylene groups and is virtually inactive when n (Table V) is greater than or equal to 7, while monoamine oxidase has its maximum affinity for diamines at n=11 (333, 335). Kenten and Mann (1952) reported that putrescine and cadaverine, of all substrates tested were most readily oxidized by pea DAO. Also oxidized were agmatine, 1,6-diaminohexane, histamine, and such phenylethylamines as β-phenylethylamine, di-β-phenylethylamine, tyramine and tryptamine (336). The pH optima were different for each substrate. Activity was negligible with 1,3-diaminopropane and maximal with putrescine. The variation of pH optimum and activity was considered to be related to their ionization, but all of these amines had very similar titration curves up to pH 9.7 and all were completely ionized below pH 8. Little velocity change in the reaction was observed with changes in diamine concentration, but β-phenylethylamine did have an

Table V. Common Diamine Oxidase Substrates

Diamines

$NH_2CH_2(CH_2)_nCH_2NH_2$

n=2 putrescine; 1,4-butane diamine; tetramethylene diamine; 1,4-diamino-butane

n=3 cadaverine; 1,5-pentane diamine; pentamethylene diamine; 1,5-diaminopentane

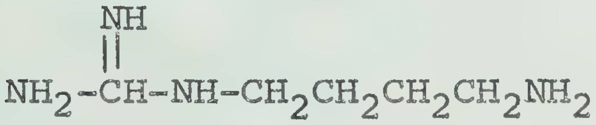
Spermine

$NH_2CH_2CH_2CH_2NH(CH_2)_4NHCH_2CH_2CH_2NH_2$

Spermidine

$NH_2CH_2CH_2CH_2NHCH_2CH_2CH_2CH_2NH_2$

Agmatine



$NH_2\text{-}\overset{\overset{NH}{\|}}{CH}\text{-}NH\text{-}CH_2CH_2CH_2CH_2NH_2$

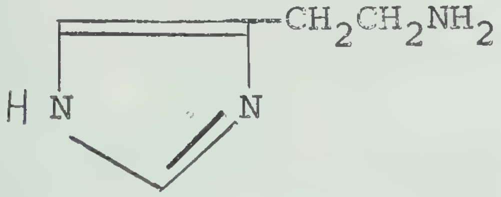
Cystamine

$NH_2CH_2CH_2\text{-}S\text{-}S\text{-}CH_2CH_2NH_2$

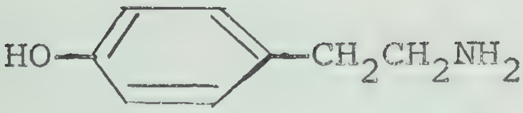
Lanthionamine

$NH_2CH_2CH_2\text{-}S\text{-}CH_2CH_2NH_2$

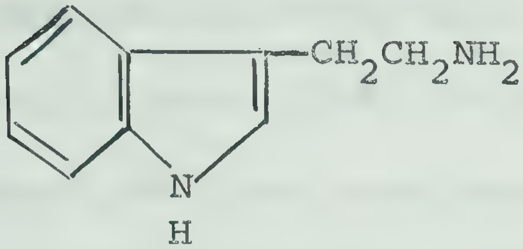
Histamine



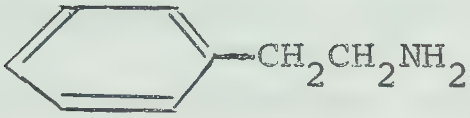
Tyramine



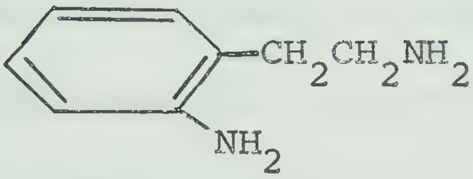
Tryptamine



β-Phenylethylamine



2-(2-aminophenyl)-ethylamine



optimum concentration. They also reported some reaction with ethanolamine, ethylamine, allylamine and n-heptylamine and with L-lysine (336). These results were later confirmed by Mann (1955) who also observed no oxidation of 1,3-diaminopropane and slow oxidation of butylamine as well as of lysine and ethylamine (389). Mann and Smithies obtained evidence for the formation of cyclic compounds from the DAO oxidation products of D,L, and DL-lysine. There was little observable difference between the reactions of the enzyme with the different enantiomers (331). The conformation about the carbon adjacent to the amino group is apparently not important. Lupine DAO has been reported to oxidize DL-lysine (45 µl O_2/30 min./mg. protein as opposed to a rate of 580 with cadaverine as substrate) (391). This preparation did not oxidize L-lysine. Hasse (404) has discussed the possible importance of the ability of DAOs to oxidize lysine in studies of alkaloid biosynthesis. Indole is formed when DAO oxidized 2-(2-aminophenyl)-ethylamine (332).

Werle and his coworkers found purified pea DAO had high activity towards cadaverine and appreciable activities towards spermidine and tyramine (378). (It should be noted that in some cases the substrate may have been acted upon by another, contaminating, enzyme. This possibility was shown by Suzuki who presented evidence that lupine extracts probably oxidized β-phenylethylamine via DAO, but tyramine by the

action of a tyrosinase (405).) Purified pea DAO also oxidized spermine and β-phenylethylamine, and had low activities towards histamine, dopamine, tryptamine, noradrenalin and serotonin. There was no activity with 6-hydroxyserotonin nor with adrenalin (378). Hill and Mann (1964) reported extensive data on the activity of pea DAO with different substrates (337). Activity was maximal with C4 and C5 diamines and was negligible with C2 and C3 diamines. The activity towards aliphatic monoamines increased with chain length from C1 to C3 and then remained relatively constant. The enzyme's affinity for substrate was less for monoamines than for diamines (337). The binding of substrate to DAO causes a change in the absorption spectrum of the enzyme. Such changes are more fully discussed below. Aniline, diethylamine and other compounds structurally related to DAO substrates caused no change in the enzyme's spectrum. However, 1,2-diaminoethane and 1,3-diaminopropane did cause a colour change that was assumed to show the formation of an enzyme-substrate complex. This complex was sufficiently stable to prevent further reaction in the case of these two diamines (337).

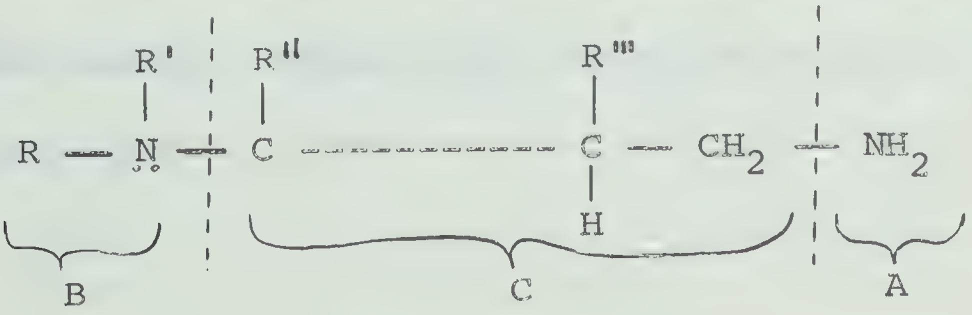
Lupine DAO oxidizes putrescine, cadaverine, agmatine, 1,6-diaminohexane, bis(5-aminopentyl)amine, tyramine, histamine, tryptamine and benzylamine in decreasing order of activity as substrates (391). DL-Ornithine and 1,3-diaminopropane were not oxidized.

Cavallini, Rotilio, and Mondovi demonstrated that DAOs were able to degrade certain sulphur-diamines (406). Hog kidney DAO oxidized cystamine at the same rate it oxidized cadaverine (406, 407) while N-acetyl cystamine was not oxidized. Similar results were obtained with pea DAO. Lanthionamine was the best sulphur diamine substrate while lanthionamine sulphone ($NH_2CH_2CH_2-SO_2-CH_2CH_2NH_2$) gave depressed activity and cystamine disulphoxide ($NH_2CH_2CH_2-SO-SO-CH_2CH_2NH_2$) abolished activity (407). It was shown later that only DAO, not MAO could oxidize sulphur diamines (408). For each molecule of cystamine oxidized about 3 atoms of oxygen were used and 2 moles of NH_3 were formed as opposed to the use of 2 oxygen atoms and production of one molecule NH_3 when cadaverine was oxidized (406). A two carbon fragment was observed to be one of the reaction products. It has been recently stated that cystamine oxidation in rat liver was carried out by enzymes with active sulphydryl groups (409). This observation, however, does not necessarily disprove earlier theories that held cystamine to be oxidized by a DAO in liver, as the study was made only on a crude system. The results with sulphur diamines indicate that the separation of the amino groups may be important to the substrate binding, but the hydrocarbon nature of the chain is not. Bulky polar groups in the chain did tend to reduce

the activity. The authors did not consider whether the effect was on the substrate binding, or on the actual reaction.

In 1965 Macholán reported that plant and animal DAOs oxidized the 2-hydroxy derivatives of cadaverine and putrescine with nearly the facility that it oxidized the parent compounds (397). More recently the activities towards (DL)-hydroxydiamines have been found to be lower than those towards the parent diamines for pea and pig kidney DAOs (398). In the presence of catalase, one mole of hydroxydiamine consumed one atom of oxygen with the formation of one mole of cyclic ω-aminoaldehyde. Hydroxycadaverine gave hydroxy-Δ^1-pyrroline which spontaneously converted to pyrrole (398). Hydroxydiamines are thought to exist naturally, and as substrates of diamine oxidase could give rise to some types of alkaloids (398, 410).

Zeller, Fouts, Carbon, Lazanas, and Voegtli (411) tested 62 diamines as possible DAO substrates and found 22 to be active. They found that the movement of the amino group from one ring position to another could abolish activity towards certain heterocyclic amines. N,N'-and α, α'-substituted diamines were not active as substrates. From this evidence they propose a three-point binding theory:



$$\underbrace{R - \overset{R'}{\underset{\cdot\cdot}{N}}}_{B} \;\Big|\; \underbrace{\overset{R''}{C} \cdots\cdots \overset{R'''}{\underset{H}{C}} - CH_2}_{C} \;\Big|\; \underbrace{NH_2}_{A}$$

The two basic sites on the molecule perform different functions - A must be a primary amine and must be attached to a methylene group. Group B is less restricted - it may be methylated or be part of a guanidine residue, however if it is trimethylated, substrate properties are lost. Group B may also be substituted for by nitrogen-containing aromatic systems. The length of the C chain has a considerable effect - this is not simply an effect of the number of methylenes separating A and B. It has been found that 1,3-diaminopropane has little substrate activity while 1,3-diaminobutane and 1,3-diaminopentane are readily degraded (329). The evidence of other workers tends to support these conclusions on substrate binding.

Fouts, Blanksma, Carbon and Zeller state that at least one of the two basic groups of DAO substrates does not interact with the enzyme by virtue of electrostatic forces and thus the substrate capabilities of certain monoamines was explained. A second amino group, however, greatly increases the affinity of the substrate for the enzyme. Possibly this second basic group serves as a nucleophile. As the nucleophilic properties are lost in N-trimethylcadaverine it is both expected and found to behave as a monoamine. N-substituted monoamines and N,N'-substituted diamines would not serve as substrates (412).

Lindell and Westling considered the degree of ionization of the two basic groups to be a factor in determining histaminase affinity for certain compounds related to histamine. The pKa values indicated that compounds oxidized by amine oxidase existed, under the reaction conditions, as monocations (413). This tends to confirm the suggestion of Fouts, Blanksma *et al.*. Lazanas (365) has reported further evidence for the functioning of the second nitrogen of diamines as a nucleophile rather than as a positively charged centre (365).

One of the amino groups of the diamine substrate (*e.g.* cadaverine) can be replaced with non-basic residues such as halogen, methyl, or hydroxyl with activities 39, 26 and 59% of that of cadaverine (412). *Para* and *meta* xylene diamines are substrates for plant and animal DAO (347).

Both plant and animal diamine oxidases have been shown to oxidize diamines, polyamines, monoamines, phenylalkylamines and certain amino acids. The oxidation of aliphatic diamines is dependent upon chain length and is maximal at C4 and C5. Neither the plant nor animal enzyme will oxidize secondary amines while monoamine oxidase will attack both primary and secondary amines (337, 412). Sulphur diamines and 2-hydroxydiamines are also attacked. The evidence tends to support Zeller's proposals that one amino group acts

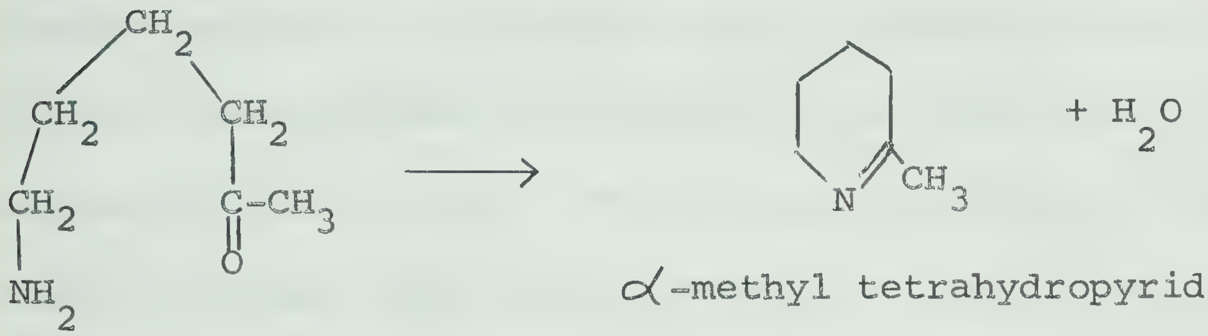
by virtue of its positive charge while the other may act as a nucleophile. Both groups are necessary for full activity, however some activity is present towards compounds with single unsubstituted amino groups. The separation of the amino groups is of importance as is the overall length of the hydrocarbon chain. A two or three point substrate binding has been proposed and at least two different types of bonding have been experimentally demonstrated (414). Substituents on/in the hydrocarbon chain appear to have little effect; further the optical configuration of the substrate is not important, at least in some cases. The chain length factor is possibly related to bonding to the enzyme, perhaps by hydrophobic forces, although the lack of effect of polar substituents on the chain tends to preclude this idea. However, the evidence available is insufficient to permit any firm conclusions. There are no reports in the literature on the use of apolar bond disrupting agents or on the use of monoamines substituted with other nucleophilic groups to make them possible diamine analogues for the study of DAO.

(b). products of the diamine oxidase reaction

The product of the diamine oxidase-catalysed oxidation of a diamine is an amino aldehyde.

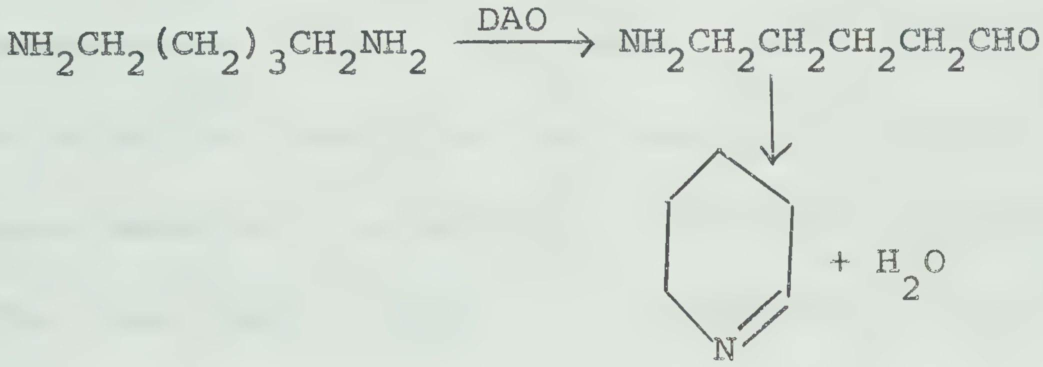
$$NH_2CH_2(CH_2)_nCH_2NH_2 + O_2 + H_2O \rightleftharpoons H_2O_2 + NH_2CH_2(CH_2)_nCHO + NH_3$$

Amino aldehydes may exist either in straight-chain form as such, or as the cyclic products formed by spontaneous elimination of water, or an equilibrium between these two forms may exist (331). The cyclization reaction is essentially a Mannich reaction between a primary amine and a carbonyl compound in a manner similar to the spontaneous cyclization of γ- and δ- methylated amino ketones (415).



α-methyl tetrahydropyridine

In the case of δ-aminovaleraldehyde:



$$NH_2CH_2(CH_2)_3CH_2NH_2 \xrightarrow{DAO} NH_2CH_2CH_2CH_2CH_2CHO$$

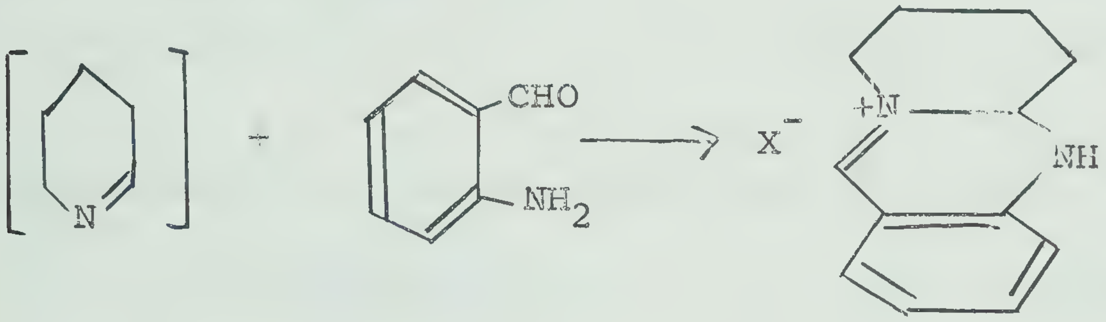
Δ'-piperideine tetrahydropyridine

The cyclization reaction is spontaneous and very rapid. The aminoaldehydes formed from putrescine and cadaverine respectively form Δ'-pyrroline and Δ'-piperideine (331). These unsaturated cyclic products probably exist in equilibrium with the open-chain forms (31) but the cyclic form is very heavily favored, especially for six- and five-

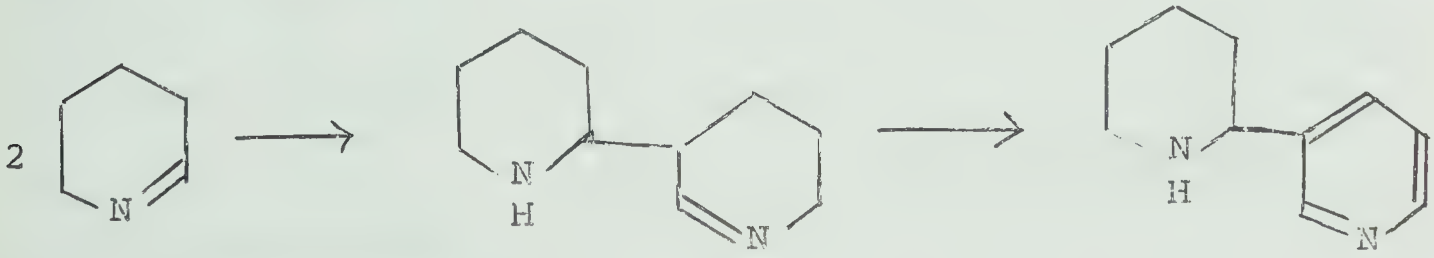
membered rings. The aldehyde nature of the initial product has been amply demonstrated and aldehyde derivatives have been isolated. There is no evidence for an aldimine product as has been proposed by Mann and Smithies (331) (see following discussion on mechanism). The first suspicion of the nature of the reaction products from C5 and C4 diamines arose from the observation that the carbonyl products of the action of diamine oxidase on cadaverine and putresine did not give reliable results when determined by an iodine-bisulphite method (331, 336, 416). The product was found to bind iodine directly rather than reacting with the bisulphite, although this uptake of iodine did not occur when the substrate was 1,10-diaminodecane (331). The direct iodine uptake was proposed to be the result of the reaction of iodine with the unsaturated heterocyclic Mannich condensation products (331, 416). The long chain length of 1-aminodecanal would tend to disfavor the cyclization and in dilute solution the free amino-aldehyde would exist, which would not react with iodine directly. Mann (331) has presented confirmatory evidence for this as the oxygen uptake when 1,10-diaminodecane is the substrate for diamine oxidase rises to values near that for the oxidation of two amino groups per mole.

The cyclic Schiff bases Δ'-pyrroline and Δ'-piperideine are very labile (358). Δ'-piperideine itself

has not been isolated, although piperideines with double bonds distant from the nitrogen have been (417). Dimers and trimers of Δ'-piperideine form spontaneously (418). Monomers of Δ'-pyrroline and Δ'-piperideine may be trapped as 1,2-dihydroquinazolinium compounds by their reaction with o-aminobenzaldehyde (419).

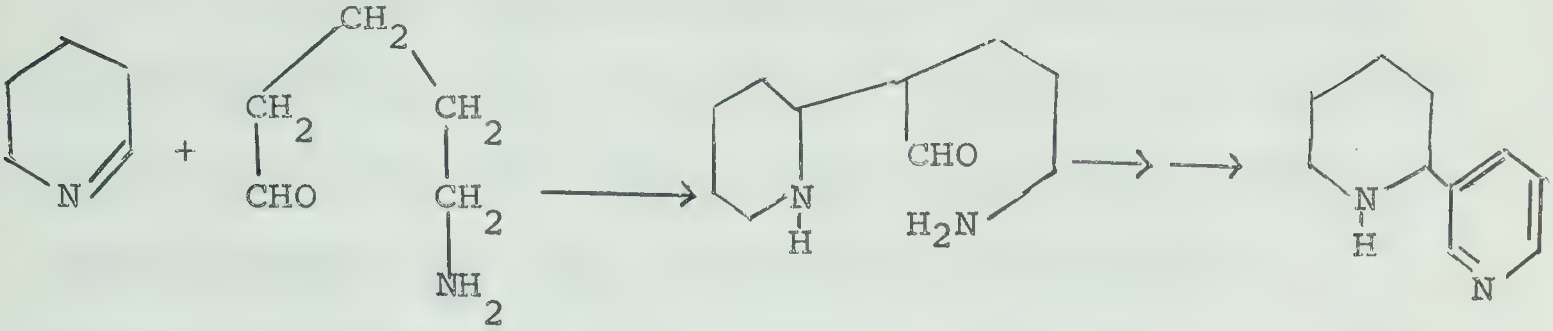


Even at zero degrees at pH 7 an aldol-type reaction converts Δ'-piperideine to a dimer and the dimer may undergo further oxidation at pH 7. The dimer produced is tetrahydroanabasine, which auto-oxidizes under the DAO reaction conditions (420, 421).

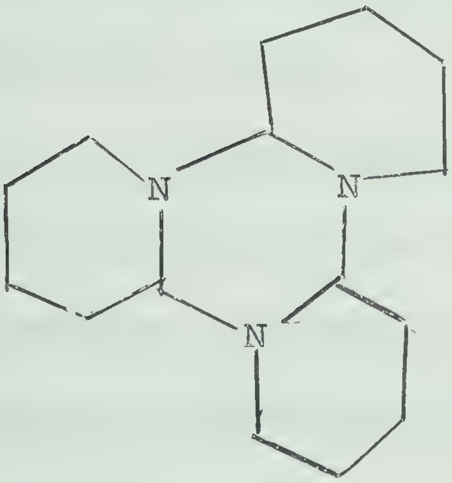


The product, the alkaloid anabasine, has been identified in enzyme reaction mixtures (421, 422). Anabasine could also

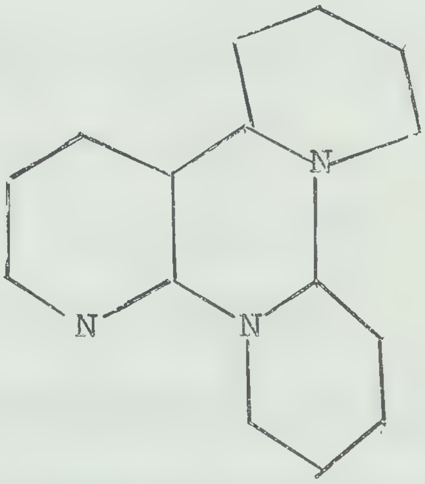
form through the condensation of a molecule of piperideine with δ-aminovaleraldehyde (31).



Piperideine also readily trimerizes to either α-tripiperideine or to *iso*tripiperideine (331, 423). These compounds (either or both) have been identified in the reaction mixtures after cadaverine oxidation by pea DAO (331).



α-tripiperideine

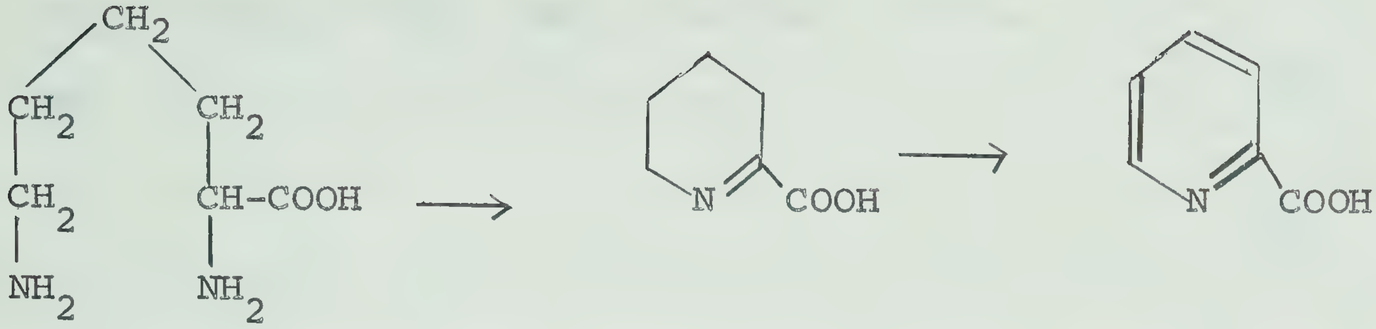


iso-tripiperideine

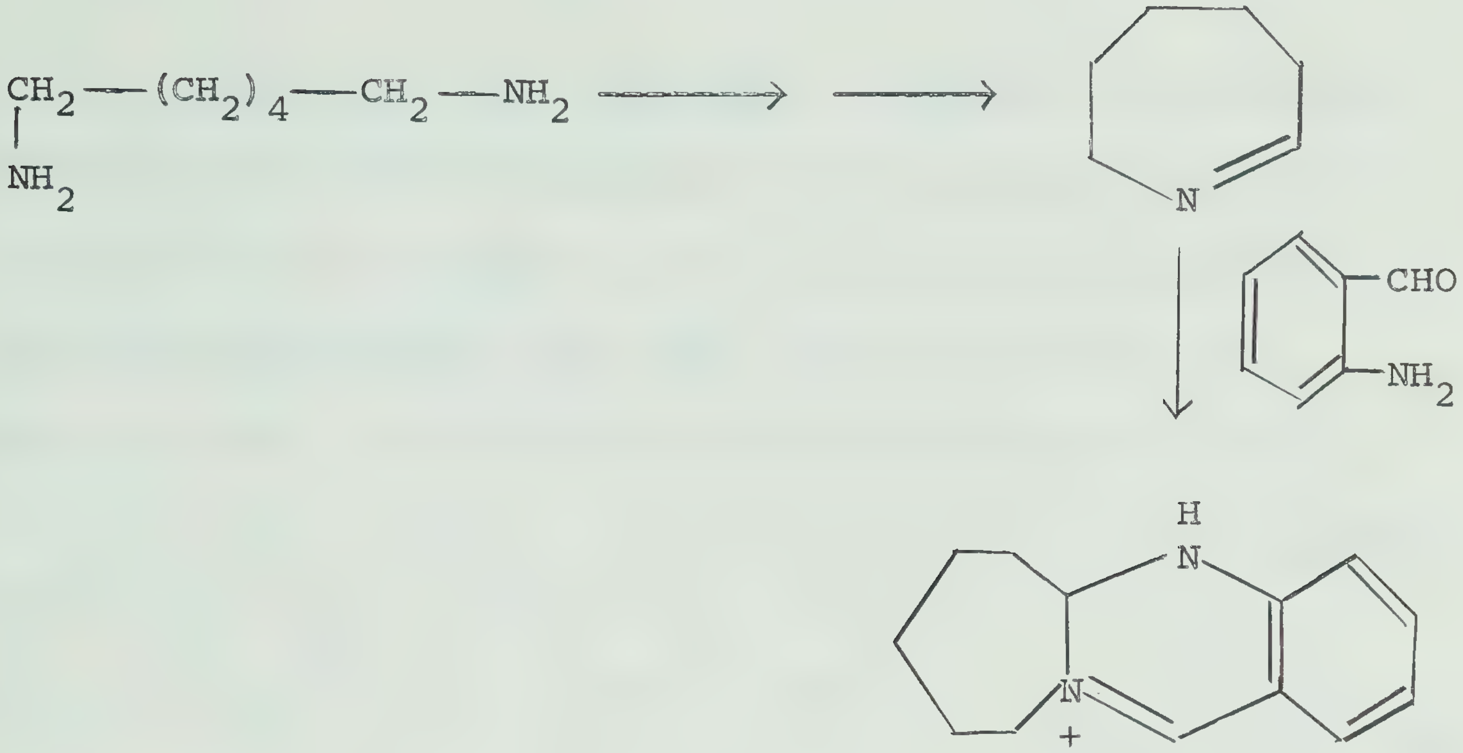
Anabasine can be formed chemically from α-tripiperideine (421). At acid pH (near 5) these trimers exist in equilibrium with the monomer and thus 1,2-dihydroquinazolinium derivatives can be made (331, 358). Other derivatives have

also been isolated (358). The tripiperideines and the dimers are able to react with Dragendorff's reagent (424). The yellow or orange coloured products of the reaction with *o*-aminobenzaldehyde form the basis of a technique for assay of DAO activity (419, 425) and are used as detection reagents in chromatography (331, 426). Care must be employed in chromatography of the tripiperideines, since heating (as often employed to dry spots) has been shown to cause formation of a series of Δ^1-piperideine polymers (424). The mixtures of cyclic products obtained through the DAO oxidation of putrescine and cadaverine can be extracted quantitatively into toluene and this property is employed in a radioassay procedure for DAO activity determination that employs labelled substrates (426).

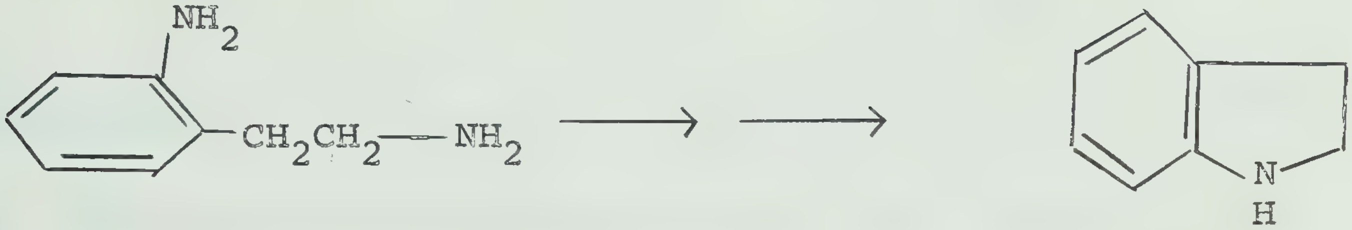
Other cyclic secondary products have been shown to form in the reaction mixture when DAO oxidizes certain substrates. Diamine oxidase-catalysed oxidation of D and L lysine has been shown to yield 3,4,5,6-tetrahydropicolinic acid that can be reduced to picolinic acid (331, 427) under biological conditions.



This compound has been demonstrated in many plants, some of which have not been shown to possess DAO activity (331). Smithies and Mann consider the possibility that DAO is involved in the initial stages in the formation of some ring-containing compounds such as various piperidine and pyrrolidine alkaloids (331). Tetrahydropicolinic acid and 5-carboxyl- Δ'-pyrroline have been formed by the action of Neurospora dehydrogenase preparations on α-amino-δ-hydroxyvaleric acid (pentahomoserine) and α-amino-ϵ-hydroxycaproic acid (hexahomoserine) (427). The cyclic compounds exist in equilibrium with the corresponding open chain semi-aldehydes that have been suggested as intermediates in the anabolism and catabolism lysine in fungi and some yeasts (428). The action of pea DAO on N-methylated cadaverine has been shown to give a mixture of products including N,N'-dimethyl- Δ^2-tetrahydroanabasine (429). The product of DAO oxidation of hydroxyputrescine is a hydroxy- Δ'-pyrroline (397) that spontaneously forms pyrrole (398). The enzymatic oxidation of hexamethylenediamine produces Δ'-azacycloheptene, that was isolated as the 2,3-pentamethylene-1,2-dihydroquinazolinium salt (430).



Pea diamine oxidase produces indole from 2-(2-aminophenyl) ethylamine (332).

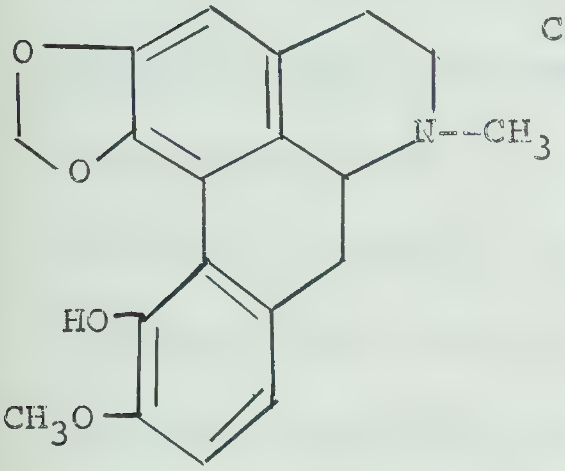


(c). Inhibitors of diamine oxidase

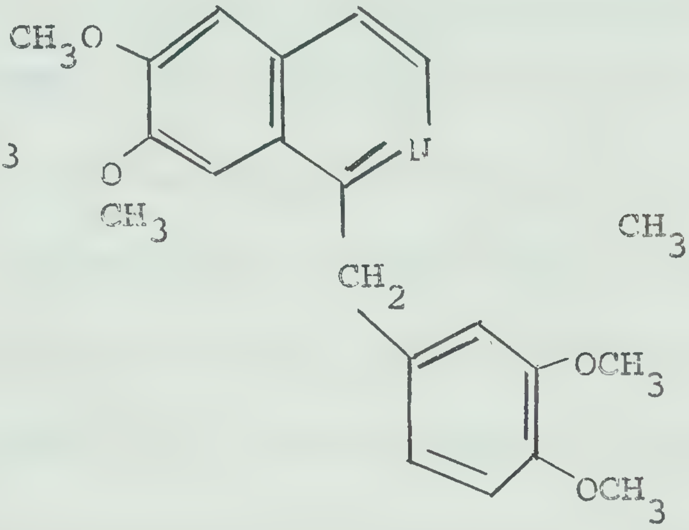
Inhibitors have given much information on the nature of DAO active groups and are of value in differentiating DAOs from MAOs. Buffoni (325) has given a good discussion of amine oxidase inhibitors.

Compounds able to react with carbonyl groups do inhibit DAOs (337, 390, 433, 436, 437, 438). Some such compounds are cyanide, semicarbazide and its derivatives, and hydrazine and hydrazides. Inhibition by riboflavin antagonists has been reported by one worker (433) but no

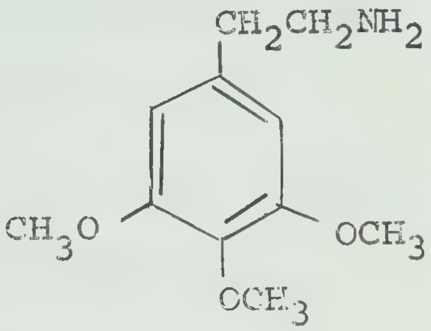
further evidence has been published. Certain other inhibitors have been found such as methylene blue and other dyes (which interfere with the redox reaction of the enzyme) and the alkaloids bulbocapnine (325, 439), papaverine (325) and mescaline (440). Bulbocapnine is a competitive inhibitor.



bulbocapnine



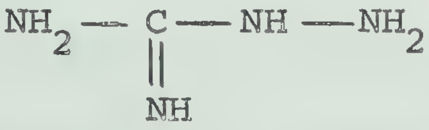
papaverine



mescaline

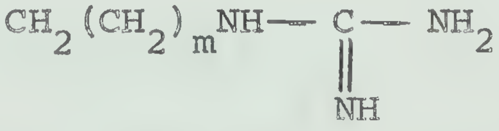
Some aminoquinoline derivatives inhibit also (325).

Aminoguanidine is a powerful DAO inhibitor (which may inhibit competitively, (325, 433) although it is a carbonyl reagent (325) and some other guanidine derivatives and diamine analogues inhibit as well (325, 433, 436, 437, 441)). In fact, aminoguanidine acting on DAO is one of the most powerful in vitro inhibitors known (437).

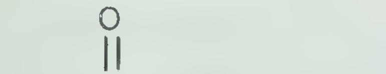


$$\mathrm{NH_2 - \underset{\underset{NH}{\|}}{C} - NH - NH_2}$$

aminoguanidine

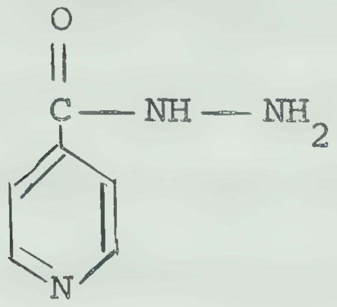


$$\mathrm{CH_2(CH_2)_m NH - \underset{\underset{NH}{\|}}{C} - NH_2}$$

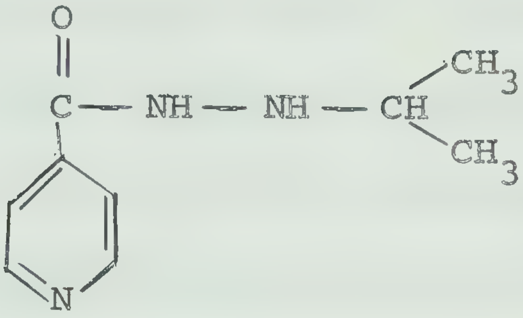
The inhibition of DAO by hydrazides has been extensively studied. Hydrazine derivatives are competitive inhibitors that frequently act on both DAOs and MAOs through their reactions with the carbonyl-group-containing amine oxidase cofactor pyridoxal phosphate (325). The L forms of amino acid hydrazides are much more effective inhibitors than their D antipodes (438). The hydrazine moiety does not necessarily have to be 'free', *i.e.* able to react with carbonyl groups, to inhibit guinea pig liver DAO (441). Thus, it appears that hydrazides may act to inhibit DAOs either as carbonyl group reagents, or in some other yet unspecified fashion (perhaps by binding at the substrate site on the enzyme as substrate analogues, thus blocking the binding of active substrates) or possibly in both modes. Semicarbazide,  $H_2N{-}C({=}O){-}NH{-}NH_2$ is another carbonyl group reagent (a hydrazide derivative) that is a frequently used DAO inhibitor which does not affect MAO (325). Certain hydrazides are able to affect both amine oxidases (325) so the particular carbonyl reagents used to differentiate between these must be selected with care.

Monoamine oxidases may be differentiated from the intracellular DAOs by their *in vitro* (and often *in vivo*) in sensitivity to semicarbazide, aminoguanidine and 'isoniazid'

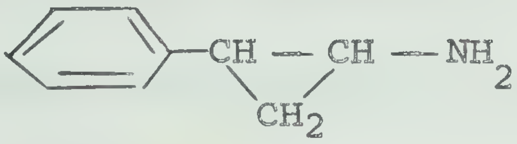
(325, 436, 441). Isoniazid (isonicotinic acid hydrazide):



inhibits DAO only, while 'iproniazid' (1-isonicotinoyl-2-isopropylhydrazine):



inhibits both DAO and MAO (325). MAOs but not DAOs are inhibited by DL-trans-phenyl-cyclopropylamine (436)



and by N-methyl-N-benzyl-2-propylamine (436). Certain inhibitors may affect both types of amine oxidase but appear to have different modes of action on each (441). In other cases inhibitors common to both are thought to show cofactor and mechanistic similarities between them. It has, for example, been proposed that isoniazid on DAO and iproniazid on MAO and DAO act by stimulating the production of free radicals in the vicinity of the active

centre catalyzed by the enzyme-bound copper (325). Caution must be exercised in extending in vitro inhibition behavior to in vivo systems (325, 436) as the potencies of the inhibitors vary greatly in some cases (442).

Pea diamine oxidase is also inhibited by sulphide (337) and slightly by azide (389). Thiourea and its derivatives do not inhibit pea DAO although they are general inhibitors of copper-enzymes (389). In some cases thiourea appears to activate the enzyme. The thiourea may protect the enzyme from the attack of the hydrogen peroxide produced in the enzyme reaction, perhaps by being oxidized itself (337, 389).

Long-chain diamines are competitive inhibitors of DAO (325). The 2-keto derivatives of putrescine and cadaverine were found to be strong competitive inhibitors of pea and pig kidney DAO although the 2-hydroxy derivatives were oxidized by the enzymes (443).

The inhibition of DAO by metal chelating agents that bind copper (325, 337, 347, 389, 390, 405, 431) will be discussed below. Divalent metal ions, including copper, inhibit pea DAO (431).

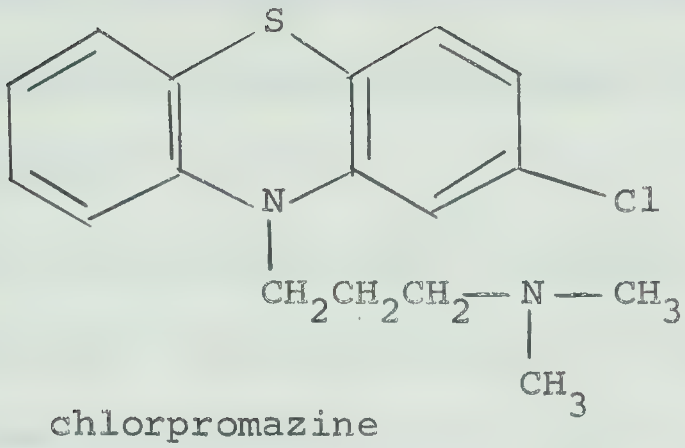
DAOs are not affected by reagents that bind sulphydryl groups (336, 432, 433, 434, 435).

Lupine diamine oxidase is inhibited by copper ions, copper chelating agents, isoniazid, cyanide, and semicarbazide (391) and is considered to be identical to the enzyme from peas. Monoamine oxidase is inhibited by the quinolizidine alkaloid sparteine (445) while DAO is not.

In the absence of catalase, hydrogen peroxide accumulates in DAO reaction mixtures (336). Pea DAO is inactivated during its reaction with the substrate (339, 431) and this inactivation is thought to be the result of the hydrogen peroxide attacking the reduced form of the enzyme (389). This inactivation occurred only when substrate was present and occurred at substrate concentrations as low as 3.3×10^{-5}M. A hydrogen peroxide-utilizing system is thus necessary for the proper functioning of DAO. Even in the presence of catalase the enzyme is inactivated if CuII ions are present (431). It was proposed that this was the result of copper-catalyzed attack of hydrogen peroxide on the enzyme and this explained the inhibition by copper ions. Further studies by the same authors, published in 1964, tend to disprove this hypothesis and they now feel that CuII causes an abnormal breakdown of the enzyme-substrate complex with subsequent damage to the enzyme. They suggest that hydrogen peroxide also acts by reaction with the enzyme-substrate complex (337). The observation that hydrogen peroxide acts

only on the substrate-reduced enzyme was confirmed with purified pig-kidney DAO (435). The inactivation was most pronounced at neutral rather than alkaline pH, suggesting that the action of the hydrogen peroxide might possibly be related to the ionization of some group on the enzyme and it is also suggested that a conformational change in the enzyme might unmask functional groups for reaction with hydrogen peroxide.

The destruction of cadaverine (and in some cases of other substrates) by clover DAO is activated by polyphenols such as noradrenalin, adrenaline and pyrocatechol as well as by glutathione and ascorbic acid. The oxidation of cadaverine is activated by α-ketoglutaric acid and by 3,4-dioxyphenylethylamine but that of histamine is inhibited! The effects of ascorbic acid and of pyrocatechol are mediated by a thermolabile, high molecular weight intermediate present in clover extracts (347). Chlorpromazine (2-chloro-10-(3-dimethylaminopropyl)-phenothiazine) has been observed to activate pea seedling DAO (444). At 10^{-3}M it produced a 250% increase in activity towards cadaverine and 455% increase in activity towards histamine.



chlorpromazine

(d). Cofactors of Diamine Oxidase

(i). metal content

Schuler and Meier reported in 1954 that divalent cations could prevent the inhibition of DAO-catalyzed cadaverine oxidation by hydrazines (446). They found CoII, NiII, CuII, and MnII were effective in preventing inhibition although they were without effect on the pre-inhibited enzyme. Ferric ions were without effect. Mann (1955) reported pea DAO inhibition by cyanide but not by azide (389), and, following previous workers, discussed the cyanide inhibition as resulting from cyanohydrin formation with the enzyme carbonyl group rather than inhibition through reaction with a bound metal. Mann also found that the enzyme was inhibited by sodium diethyldithiocarbamate (DIECA), potassium ethyl xanthate, and by salicylaldoxime - all of which are inhibitors specific for copper enzymes. He reported that the enzyme suffered inactivation during the reaction, a property that

is characteristic of copper-containing oxidases. The partially purified preparations employed had copper and manganese contents of 0.03% to 0.06%.

Werle and Hartung (347) confirmed the inhibition of plant DAO by copper chelating agents,—8-hydroxyquinoline, DIECA, salicylaldoxime and dithizon were shown to inhibit both clover and kidney DAOs. Suzuki added α, α'-dipyridyl and o-phenanthroline to the list of metal complexing agents known to inhibit plant (pea and lupine) DAOs (405). He concluded that possibly Mo, Fe or Zn were involved, but did not mention Cu, which is also a possibility in view of the data he presented.

Mann further studied the metal content of pea seedling DAO by spectrographic and colorimetric methods (390). He found the content of Mn and Cu to be highest while the Zn and Fe contents were lower although still at significant levels. In the initial preparations Mn was higher than Cu but on further purification the copper content increased to 0.08 to 0.09% while the manganese content dropped to 0.01%. In a purified preparation zinc was less than 0.06% and other metals were below 0.004%. Dialysis of the purified, pink enzyme against DIECA solution resulted in a rapid colour change to yellow followed by sedimentation of a yellow precipitate - that left the solution again pink. The precipitate appeared to be Cu-DIECA and accounted for 90% of the copper present

initially. This treatment inactivated the enzyme and CuII treatment was able to partially restore the activity. Zinc ions had no activating effect on the DIECA-dialyzed preparation.

Werle *et al.* demonstrated CuII or ZnII reactivation of plant DAO inhibited by 8-hydroxyquinoline (378). Zinc also reactivated DAO after inhibition by *o*-phenanthroline. A specific zinc binding agent, however, did not affect enzyme activity and FeII or FeIII ions did not affect the activity of the inhibited enzyme. In many cases these workers found that zinc and copper ions had the same effects.

Results similar to Mann's were obtained by Yamada and Yasunobu (393) with crystalline plasma amine oxidase. Removal of CuII with DIECA resulted in loss of enzyme activity that was restored by CuII treatment. They found no other metals present in significant amounts and found no valence change in the copper under anaerobic conditions.

Hill and Mann (431) studied the inhibition of pea-seedling DAO by metal chelating agents more exhaustively and demonstrated that the degree of inhibition of the enzyme was directly related to the copper-complexing properties of the reagent. They pointed out that potassium ethyl xanthate was a better *specific* agent for copper than was DIECA. The possibility that molybdate was involved was dismissed after the demonstration that molybdate was without effect on the

inhibited enzyme. The inhibition by some of the chelating agents could have resulted from binding of molybdate rather than of copper. It was found that there was no change in the valency of the copper during the enzyme reaction. Cyanide was suggested to inhibit by reacting with the copper rather than acting as a carbonyl reagent (431).

It was thought possible that the inactivation of DAO during the reaction was the result of the hydrogen peroxide produced being involved in some copper-catalyzed reaction.

Spectrophotometric studies have shown pig kidney DAO to be a cuproprotein (447, 448). Goryachenkova and Ershova (449) reported inactivation of kidney DAO by copper-chelating agents, but the reactivation of the enzyme by metal ions was only slight. Their preparation had a copper content of 0.03% (449). Hill and Mann, using a more purified pea DAO, found a copper content of 0.087% (337). This represented one gram atom of copper per 73,000 grams of protein (the molecular weight of this preparation was 96,000 from ultracentrifuge and electron microscope studies). DIECA caused the absorption spectrum of pea DAO to change to a spectrum characteristic of Cu-DIECA. The copper-free enzyme was reactivated by CuII while molybdate, CaII, MgII, FeII, FeIII, CoII, NiII, and ZnII were ineffective (337). The purified clover DAO of Werle, Trautschold and Aures contained 0.12% copper and 0.05% iron (378).

Pig kidney DAO purified 1700 fold had a copper content of 0.06% (340). The effect of DIECA on this preparation was that typical of a copper-containing protein. This hog kidney enzyme, however, could not be freed of copper by DIECA treatment. Goryachenkova and Ershova have recently confirmed their observations on the copper content of hog kidney DAO (450). A more highly purified kidney DAO had a copper content of 11 to 12 mμ moles Cu per mg. protein (395). Treatment with DIECA removed activity and CuII treatment restored it, although excessive amounts of cupric ions were inhibitory. Recent electron-spin resonance ('ESR') studies have failed to show a valence change in copper during the enzyme reaction (393, 451). ESR studies show the copper ions to be in an environment of tetragonal symmetry and all of the cupric ions are equivalent (395). Jørgensen (452) has reviewed the chemical bonding of copper and the interpretation of ESR spectra. The data showing the absence of a change in copper valency during the reaction are based on the absence of a signal, rather than its presence. Such data are indicative but not unequivocal.

Aspergillus niger amine oxidase (crystalline) contained 3 gram atoms of copper per mole of enzyme (1 g. atom per 83,000 g. protein) (453). Dialysis against DIECA inhibited the enzyme and CuII ions reactivated it. No valence change in the copper could be observed. Monomethylamine

oxidase (from fungi - not a DAO) had a copper content of 1 g. atom per mole (454). Beef liver mitochondrial monoamine oxidase is also a cupric copper containing enzyme (455).

It appears valid to conclude that diamine oxidases (indeed, perhaps all amine oxidases) are copper containing enzymes in which the copper is tightly bound (although the relative strengths of this binding vary with preparations from different sources). DAOs are inhibited by copper-complexing agents. The copper acts in the cupric form, and no valence changes or cuprous copper have been detected. The involvement of other metals does not appear likely.

(ii) pyridoxal phosphate as a diamine oxidase prosthetic group

The presence of an aldehyde residue in clover extracts with DAO activity was first postulated in 1938 (329). In 1947 plant diamine oxidase was shown to be inhibited by carbonyl reagents (341). Werle and Pechmann (339) demonstrated in 1949 that the carbonyl group of the enzyme (as shown by the action of specific inhibitors) might be that of pyridoxal phosphate, as addition of pyridoxal hydrochloride stimulated the action of crude clover DAO on cadaverine. Cafiero found that the action of placental DAO was not stimulated by pyridoxal phosphate addition (456). Kenten and Mann found that pea DAO was inhibited by reagents specific for carbonyl groups and proposed that cyanide inhibition was the result of cyanohydrin

formation with a necessary carbonyl group (336). The possibility that cyanide inhibition could also be the result of action with the bound copper was discussed in section (i). Pyridoxal phosphate enhanced the action of *Pseudomonas aeruginosa* amine oxidase (354). Carbonyl group reagents are known to inhibit *Aspergillus niger* amine oxidase (453) and fungal monomethylamine oxidase (454). Goryachenkova could not demonstrate a role for pyridoxal phosphate in model systems based on proposed reaction mechanisms. But he was unable to show that it was or was not involved in the actual enzyme reaction (433). He later observed an increase in activity of dialyzed DAO when pyridoxal was added (432). Rats suffering from vitamin B6 deficiency had DAOs that were markedly reduced in specific activity; the activity of the enzyme isolated from these animals was increased by pyridoxal phosphate addition (432). The holoenzyme (from either hog kidney or pea seedlings) was not reversibly separable into apo- and co-enzyme. The loss of activity resulting from attempts at separation was shown to be the result of the high lability of the apoenzyme towards actinic light and dilute acid media (432). Davidson has also presented evidence for the involvement of pyridoxal phosphate in diamine oxidases, although he too was unable to separate it from the apoenzyme (457).

Suzuki (1959) again suggested that cyanide inhibition arose from cyanohydrin formation with an essential carbonyl group (405). He was able to demonstrate pyruvate reversal of cyanide inhibition. Goryachenkova reported that boiled pea extract, pyridoxal phosphate and FAD activated pea diamine oxidase (458). Highly purified plant DAO gave no hint of pyridoxal phosphate (378) and the enzyme could not be separated into co- and apo-enzymes.

Mann (1961) demonstrated a change in the absorption spectrum of pea DAO upon interaction with a diamine (390). He proposed that the original pink colour of the preparation was given by an aromatic carbonyl group. The yellow colour formed after interaction of the DAO and substrate had spectral properties similar to those of the Schiff base formed between the substrate and pyridoxal (390). He was, however, unable to show the spectrum characteristic of a pyridoxal enzyme nor was he able to release pyridoxal phosphate by methods known to be effective for other pyridoxal phosphate enzymes. Mann suggested that the prosthetic group of pea DAO was a complex of a carbonyl group and CuII, but he did not commit himself as to the presence of pyridoxal phosphate as the source of this group (390).

Yamada and Yasunobu found that copper-free plasma amine oxidase had the characteristic spectrum of a pyridoxal

phosphate enzyme (459). The enzyme was represented as protein $(CuII)_4^-$(pyridoxal phosphate)$_2$. Pyridoxal phosphate was tightly bound, but was freed upon proteolytic digestion (459). Pyridoxal phosphate antagonized the inhibition of hog kidney DAO by bulbocapnine (439). Kapeller-Adler's histaminase preparation was said to give spectral evidence for the presence of pyridoxal phosphate (460). She found that the enzyme could be activated by pyridoxal phosphate, but that the activation showed a very distinct concentration optimum - excess could even inhibit (345). Relatively crude preparations of hog kidney DAO were not activated by pyridoxal phosphate, but activation was observed with more purified enzyme preparations (448). Uozumi, Nakahara, Higashi, and Sakamoto did a kinetic study on the reactivation of hog kidney DAO by pyridoxal phosphate and obtained a K_m of $5 \times 10^{-8}M$ (364). The presence of pyridoxal phosphate could be demonstrated only when DIECA was present (340). Apparently the copper masks or changes the usual pyridoxal phosphate absorption spectrum.

In 1964, Hill and Mann found no conclusive evidence for the presence of pyridoxal phosphate in their highly purified pea DAO preparation although the presence of a necessary carbonyl group was again reiterated (337). Goryachenkova confirmed in 1965 that purified hog kidney DAO contained firmly bound pyridoxal (450). Blaschko and Buffoni,

also in 1965, published evidence that pyridoxal phosphate was a constituent of hog plasma amine oxidase (461). They were able to show this by reactivation of the apoenzyme of another enzyme known to require pyridoxal phosphate as coenzyme, by addition of a preparation of hydrolyzed plasma amine oxidase. They found three to four moles of pyridoxal phosphate per mole of enzyme. Very highly purified hog kidney DAO was shown to contain pyridoxal phosphate by spectrophotometric studies on the enzyme after treatment with carbonyl reagents, by chemical phosphorus determinations, by isolation of pyridoxal phosphate semicarbazone from the enzyme, and by use of a hydrolyzed DAO preparation to reactivate apo-aspartate aminotransferase (394). The pyridoxal phosphate content appeared to be one mole per gram atom of copper (equivalent to one molecule per 90,000 molecular weight unit). Pyridoxal phosphate was released from hog kidney DAO by repeated freeze-thawing after incubation with DIECA or phenylhydrazine (394). Denaturation by boiling, ultra violet radiation, and by acid and alkali treatment failed to release pyridoxal phosphate (394).

Thus, the involvement of pyridoxal phosphate in the action of DAOs from mammalian sources seems to be reasonably confirmed. Diamine oxidase appears to be composed of one or more units of 90,000 molecular weight, each containing an atom of copper and a molecule of tightly-bound pyridoxal

phosphate. The presence of pyridoxal phosphate in plant DAO has not been so firmly established, although, by analogy and from the evidence that is available, this seems likely. More experimental work is needed to show the presence of pyridoxal phosphate in plant DAO and to establish its role in the action of these enzymes.

(iii). flavin adenine dinucleotide as a diamine oxidase prosthetic group

The release of hydrogen peroxide during the DAO reaction lead to a proposal that flavins were involved as prosthetic groups, however concrete evidence for this has not been obtained. The issue has been further clouded by the large variety of different amine oxidases studied.

Werle and Pechmann (1949) suggested a flavin component in plant DAO but were unable to detect it (339) Kapeller-Adler found evidence for the presence of FAD and found that it activated a dialysed histaminase preparation towards histamine oxidation, but inhibited activity towards cadaverine (344). It appears possible that her preparation may have been contaminated with an FAD-requiring MAO and that the activity towards cadaverine was the result of a non-flavin DAO. Tanaka published evidence that the enzyme responsible for cadaverine and histamine deamination in *Pseudomonas* required FAD (354). Putrescine oxidase (an essential sulphydryl group -

containing amine oxidase from Micrococcus rubens) has been shown to contain FAD (352). Its susceptibility to -SH group reagents marks it as an atypical diamine oxidase.

The presence of flavin components in DAO is assumed by Goryachenkova in his discussion of possible mechanisms, but he gives no evidence for their actual presence (433). He later reported inhibition of hog kidney DAO by riboflavin antagonists and presented other evidence that DAO is a cofactor of DAO (432). Plant DAO, extensively dialyzed against acetate buffer could not be reactivated by FAD, FAD plus pyridoxal phosphate, pyridoxal phosphate, nor by boiled pea seedling extract. After prolonged dialysis against water, plant DAO could sometimes be reactivated by the boiled extract, or by FAD plus pyridoxal phosphate. The possible involvement of flavin was also shown by studies of rats deficient in vitamins B2 and B6. The addition of FAD to isolated tissues from B2 deficient rats restored the diamine oxidase activity (432, 458).

Using highly purified clover DAO, Werle, Trautschold and Aures (1961) (378) and Mann (1961) (390) were unable to find any evidence for flavin components in the enzyme absorption spectrua. It should be noted that pyridoxal phosphate was not detected in some cases unless special measures were employed, and the same might be true for flavins. There is no report in the literature of an ESR signal characteristic

of the activity of other flavin-containing enzymes. Supposedly clear spectrophotometric and fluorimetric results demonstrated that both FAD and pyridoxal phosphate were present in highly purified histaminases (346, 460). Yamada and Yasunobu were unable to detect flavins in plasma amine oxidase even after heat denaturation or pronase treatments (459). Kapeller-Adler and MacFarlane then gave further evidence - including chemical evidence - for FAD in hog kidney histaminase and assumed its presence in discussing possible reaction mechanisms (345). Their work was confirmed by other workers (364). FAD was demonstrated to be involved in the restoration of hog kidney histaminase activity and a K_m of 6×10^{-9}M was reported for FAD binding (364). Goryachenkova and Ershova (450) later reported that FAD was not involved in the activity of highly purified hog kidney DAO. Hog kidney DAO purified 3200 fold was shown by careful chemical and fluorescence studies (395) to be free of flavins.

Recently (1966) Nara and his associates have isolated from beef liver mitochondria a monoamine oxidase that they have convincingly shown to contain a flavin prosthetic group (407, 455, 462). The flavin has been shown to be covalently bound to the enzyme protein (407). This mitochondrial monoamine oxidase has an essential sulphydryl group and contains copper (407, 455). The references on

this MAO omit any discussion (or data from carbonyl reagent inhibition experiments) on the presence of pyridoxal phosphate. It is assumed that pyridoxal phosphate is absent. Native mitochondrial beef liver MAO did not have the characteristic fluorescence of a flavo-protein but a flavopeptide was found after hydrolysis (462). In a review of the literature (462) it is concluded that flavin is absent from plasma amine oxidase and from hog kigney DAO.

The existence of a flavin-containing MAO may explain the inhibition of rat liver MAO by proflavin and the drop in MAO activity accompanying vitamin B2 deficiency. This beef liver MAO is the only known example of an enzyme requiring both copper and flavin (462). Putrescine oxidase (*Micrococcus*) is not inhibited by metal chelating agents so possibly contains no copper (352). In view of the rarity of FAD-containing copper enzymes and the persistent finding of flavins in the preparations made by some workers, it appears possible that these preparations contain closely related flavin-containing amine oxidases. The DAO preparations of many workers have not been studied with inhibitors, so a MAO might be present and not be detected. The evidence for the presence of both pyridoxal phosphate *and* flavin is not so readily explained. Possibly MAO contaminated DAO preparations or possibly vitamin B2 deficiency had some variety of synergistic

effect on vitamin B6 usage (perhaps on DAO synthesis?) - these might explain Goryachenkova's nutrition studies (432, 458).

It is apparent that many further careful studies are required before the question of the presence of flavins in diamine oxidases can be properly answered. Present evidence tends to indicate that flavins are not present in typical diamine oxidases (325); at least in hog kidney DAO and in plant DAOs. Flavins are not mechanistically necessary (Hamilton - in discussion section of reference 407) and a reaction mechanism (based on model reactions) involving only a metal and pyridoxal has been proposed by Hamilton and Revesz (396). The absence of flavins has not been proved however, and their involvement remains a possibility.

(e). Kinetics of the diamine oxidase reaction

Very little kinetic data is available on the diamine oxidases although some inhibition kinetics studies have been reported. The only K_m values I have found after an extensive literature survey are for 2-hydroxyputrescine and 2-hydroxycadaverine. These are $6.3 \times 10^{-4}M$ and $1.5 \times 10^{-4}M$ for pea seedling DAO and $5.0 \times 10^{-4}M$ and $8.0 \times 10^{-4}M$ for hog kidney DAO (398). The kinetics of the diamine oxidase reaction should be studied. Spectrophotometric studies should be useful as these enzymes undergo a colour change during the reaction.

(5). Reaction Mechanisms of Diamine Oxidase

(a). Spectrophotometric studies of the diamine oxidase reaction

Few thorough spectrophotometric studies have been published on DAOs. Yamada and Yasunobu detected in the spectrum of copper-free plasma amine oxidase a maximum at 380 mμ - indicative of pyridoxal phosphate (459, 463). They also found, with the pure enzyme, maxima at 280 and 480 to 500 mμ with a shoulder at 510 mμ. The pink colour of the enzyme was related to the strong absorption at 480 mμ. Under anaerobic conditions the pink colour was bleached by the substrate, benzylamine, and by dithionite. Oxygenation restored the pink colour (463). The action of dithionite probably involved both a reduction of CuII to CuI and a reaction with the other prosthetic group to bleach the enzyme (464). In copper-free plasma amine oxidase the 480 mμ maximum is absent and a new peak at 380 mμ appears. Benzylamine had no effect on the spectrum of copper-free enzyme but dithionite did bleach it under anaerobic conditions. The restoration of activity by cupric ions was accompanied by a decrease of the 380 mμ peak and by an increase at 480 mμ (464). Partially purified preparations of hog kidney DAO were colorless (329), and only a maximum at 280 mμ was observed in their absorption spectra (340). More highly purified hog kidney DAO was 'pink-yellow' and showed maxima at 405, 480, and 277 mμ with shoulders at 260 and 290 mμ (395). The

absorption at 480 mμ vanishes after addition of substrate under anaerobic conditions. Oxygen reverses this substrate effect. The two visible maxima were decreased by dithionite treatment (395). Kapeller-Adler and MacFarlane found evidence in the absorption spectrum of histaminase for the presence of pyridoxal phosphate and for FAD (345, 346). Serratia marcescens amine oxidase is also pink, but this may be the result of a contaminating pigment (329). Fungal monomethylamine oxidase is pink (maxima at 280 mμ and 480-500 mμ) (454). Treatment of this enzyme with monomethylamine reduced the intensity of the 480 mμ band and oxygen treatment restored it. The FAD-containing, essential sulphydryl group-containing putrescine oxidase of Micrococcus rubens was yellow (maxima at 280, 380 and 460 mμ) (352). Under anaerobic conditions dithionite or putrescine discharged the colour, which could be restored by bubbling oxygen through the solution. Beef liver mitochondrial monoamine oxidase is also yellow, but in other respects its spectrum is similar to that of plasma amine oxidase (407, 455).

During the preparation of pure pea seedling DAO, Mann observed that the most active fractions were pink and that this colour was associated with a maximum at 500 mμ that was relatively weak when compared to the intensity of the protein absorption at 280 mμ (390). The colour and the 500 mμ band were discharged by dithionite and were restored by oxygen

treatment (390). Under anaerobic conditions, reaction of the enzyme with putrescine reduced the 500 mμ band and gave rise to bands at 465, 435, and 350 mμ. These bands vanished and the original colour was restored when oxygen was admitted. Mann has also observed a shift in the spectrum of pyridoxal phosphate on addition of putrescine that tends to confirm the possible involvement of this cofactor (390). Hydrazine treatment causes the appearance of a yellow colour (bands at 420 and 330 mμ). Oxygen bleaches the yellow colour, but the original pink is not restored. It was suggested that the pink compound was readily reduced and was auto-oxidizable - possibly functioning as a proton carrier. The colour change during the reaction with substrate was proposed to be part of a cyclic oxidation-reduction process. The spectral properties are taken to be suggestive of pyridoxal phosphate being involved, although the evidence is not sufficiently good to make this positive (390). Werle, Trautschold and Aures obtained results similar to Mann's (378). They found highly purified pea-seedling DAO to be rose-red, and that this colour was changed to yellow upon anaerobic treatment with substrate. The rose colour was regenerated by oxygen. The spectrum showed absorption in the 700 mμ region and a shoulder at 500 mμ.

In 1964 Hill and Mann confirmed their earlier results and found the pink enzyme was changed to yellow (with similar

spectra) by anaerobic treatment with ethylamine, benzylamine, 1,10-aminodecane, noradrenalin, lysine, histamine, spermine, and spermidine (337). In all cases the yellow colour was discharged by oxygen. The yellow form of the enzyme was hypothesized to be an enzyme-substrate complex. The formation of this complex was most rapid with those substances that were the best DAO substrates (*e.g.* putrescine) - reaction was complete within 15 seconds while reaction with L-lysine or with ethylamine was very much slower. Non-active compounds that were structurally related to the enzyme's substrates were studied. No spectral change was produced by aniline, 1-methylbenzylamine, diethylamine, *iso*propylamine, and adrenaline under anaerobic conditions. Yellow products resulted from 1,2-diaminoethane and from 1,3-diaminopropane with spectra similar to those of the enzyme-true substrate complexes, however, these yellow compounds were very much more stable to oxygen and the pink colour did not return after fading of the yellow. Hill and Mann suggest that in these cases an enzyme-substrate complex does form, but the complex is so stable that it results in inactivation of the enzyme. The behavior of diaminopropane was thus intermediate between that of putrescine and diethylamine (337).

DIECA caused the colour of pea-seedling DAO to change to orange-red and a band at 436 mμ appeared that

resembled that of Cu-DIECA (337). Little change in the spectrum resulted from treatment of the enzyme with 1,10-phenanthroline, but the formation of the yellow colour on putrescine addition was prevented. This inhibition was reversed by NiII. The yellow colour of an enzyme-substrate complex was rapidly discharged by 1,10-phenanthroline and the pink colour did not reappear (337). Copper-free pea DAO was orange-pink and CuII addition restored the pink colour of the native enzyme and shifted the maximum from 480 to 500 mμ. The colour of the copper-free enzyme was removed under anaerobic conditions by addition of putrescine, but no yellow enzyme-substrate complex formed - only a slight inflection at 466 mμ appeared in the spectrum. Preincubation of copper-free DAO with CuII and treatment with EDTA (ethylene diamine tetra-acetate) (to prevent inactivation of the enzyme by CuII during the reaction) allowed the formation with putrescine of the usual yellow complex that returned to the pink form on exposure to oxygen. Nickel gave bleaching but no colour formation with putrescine (337). The yellow colour of reduced DAO was unstable in the presence of free CuII ions. Hydrazine treatment changed the colour of the purified enzyme from pink to yellow (bands at 333 and 415-430 mμ). The latter band vanished and the 333 mμ band shifted and increased, then decreased when the preparation was allowed to stand in air.

Hydrazine treatment changed the orange-pink copper-free protein to a yellow colour. It was suggested that the hydrazine derivative of the enzyme was unstable in the presence of cupric ions (337).

The interaction of hog kidney DAO with ^{14}N- and ^{15}N-putrescine has been studied by ESR (395, Beinert in discussion section of reference 407). Replacement of ^{14}N-putrescine with ^{15}N-putrescine gave no change in the superhyperfine structure of the ESR spectrum: this indicated that an amino group of the substrate was not a ligand of copper. However, changes in the structure of the spectrum, were observed on interaction with the substrate and this might indicate conformational changes in the protein (affecting the copper) produced by the binding of the substrate.

In conclusion, most diamine oxidases are pink in colour and are reduced by their substrates to produce yellow enzyme-substrate complexes. This behavior is apparently mainly the result of the bound pyridoxal phosphate and is not directly the result of interaction with the copper.

(b). Proposed mechanisms for the diamine oxidase reaction

After the substrate has been bound to the enzyme, it must be acted upon either by the enzyme itself or by its prosthetic groups to convert an amino group to free NH_3 and its adjacent methylene to an aldehyde function with the release

of hydrogen peroxide. The stoichiometry of the reaction has been amply demonstrated to be:

$$NH_2CH_2(CH_2)_nCH_2NH_2 + H_2O + O_2 \longrightarrow H_2O_2 + NH_3 + NH_2CH_2(CH_2)_nCHO,$$

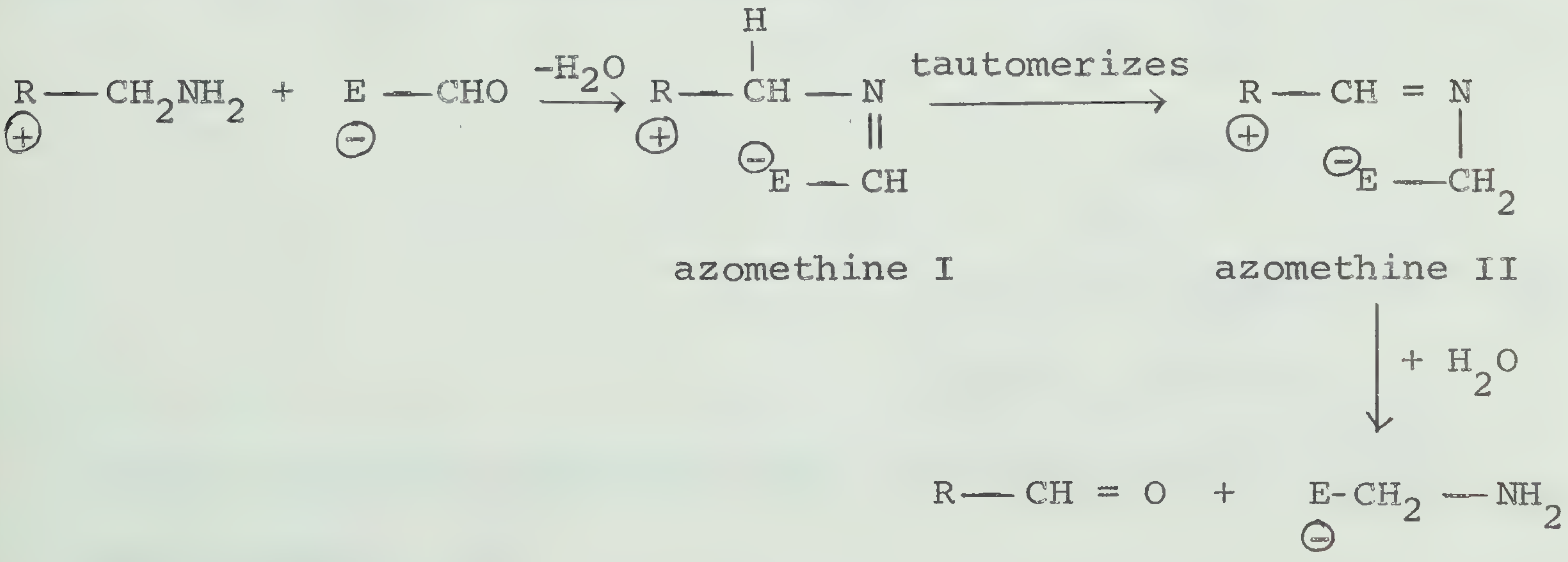
or if catalase is present:

$$NH_2CH_2(CH_2)_nCH_2NH_2 + 1/2\ O_2 + H_2O \longrightarrow NH_2CH_2(CH_2)_nCHO + NH_3 + H_2O$$

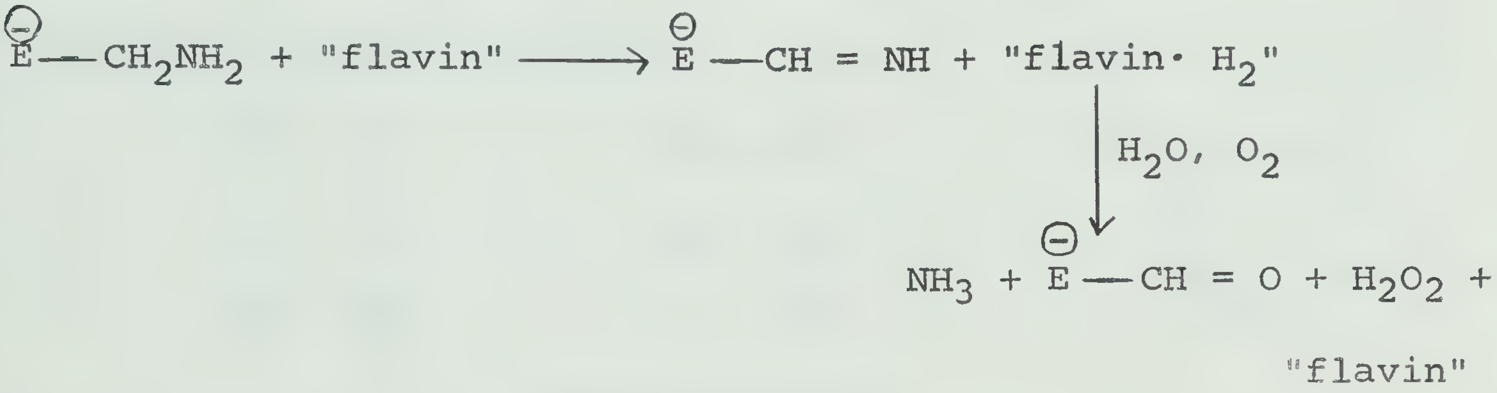
(329, 336, 339, 416, 465).

Plant diamine oxidases appear to contain both cupric copper and a carbonyl functional group that is probably that of pyridoxal phosphate. The involvement of flavin compounds is questionable and is not required for several proposed mechanisms. It has been hypothesized that copper and the carbonyl group interact, possibly as a $(CuII)_4$-(pyridoxal phosphate$)_2$ complex (340), or as a complex with the pyridoxine-bound substrate (337).

In 1949 Werle and Pechmann proposed mechanisms based on the formation of a Schiff base with pyridoxine and the involvement of flavin to release hydrogen peroxide (339).

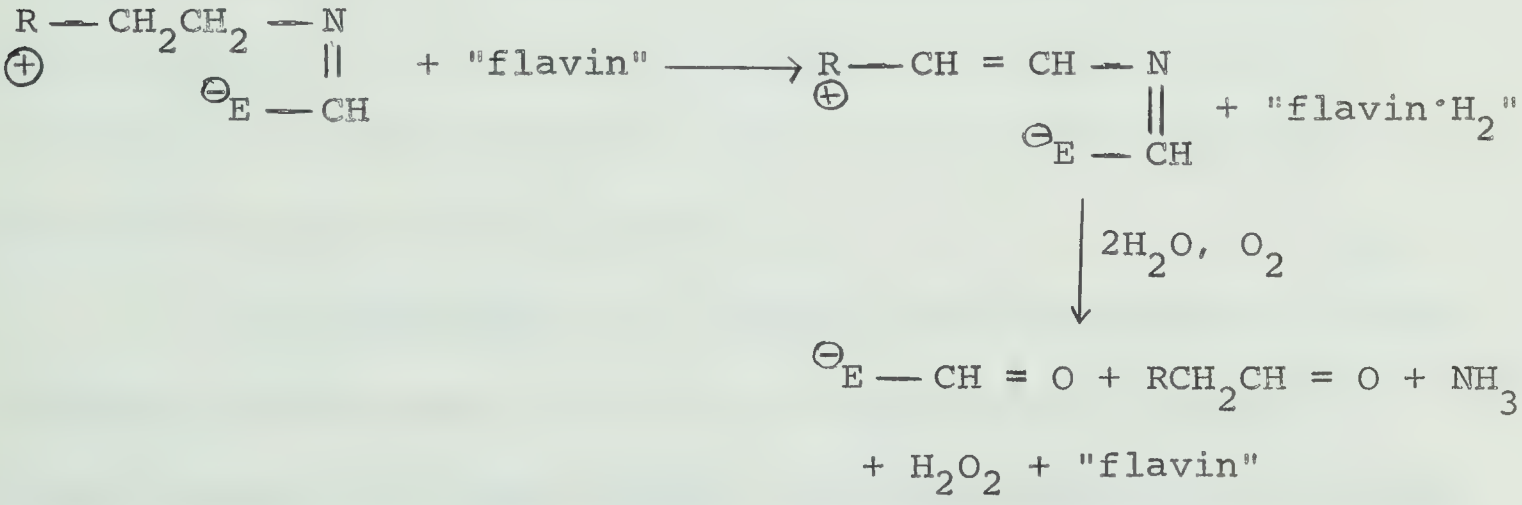


The enzyme-amine formed through the transfer of the amino group from substrate to enzyme via the Schiff base type intermediate is dehydrogenated by the action of a flavin to an imine that is hydrolyzed to free ammonia and the native enzyme (E) (339).



$$\overset{\ominus}{E}-CH_2NH_2 + \text{"flavin"} \longrightarrow \overset{\ominus}{E}-CH=NH + \text{"flavin}\cdot H_2\text{"} \xrightarrow{H_2O,\ O_2} NH_3 + \overset{\ominus}{E}-CH=O + H_2O_2 + \text{"flavin"}$$

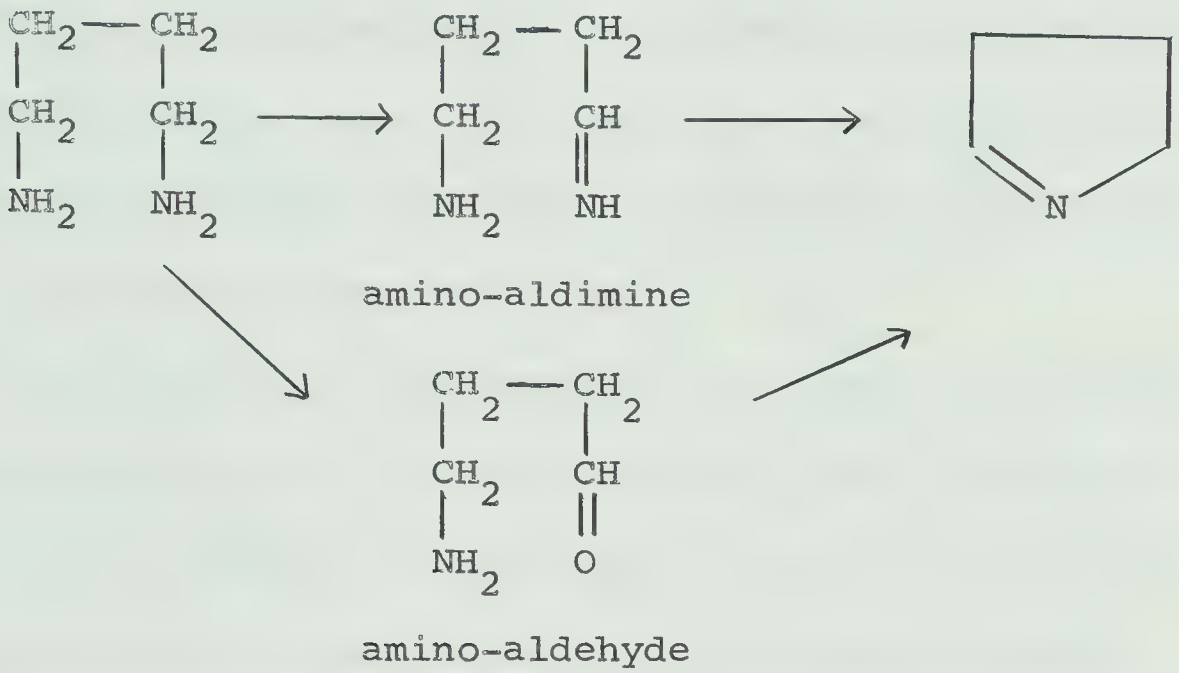
Alternatively, they propose that after the formation of the Schiff base, an enzymic dehydration may occur by virtue of the activation of the carbon atom adjacent to the Schiff base nitrogen. Hydrogenation and hydrolysis of the conjugated intermediate so formed would permit regeneration of the enzyme and liberation of ammonia.



$$\underset{\oplus}{R}-CH_2CH_2-\underset{\overset{\|}{{}^{\ominus}E-CH}}{N} + \text{"flavin"} \longrightarrow \underset{\oplus}{R}-CH=CH-\underset{\overset{\|}{{}^{\ominus}E-CH}}{N} + \text{"flavin}\cdot H_2\text{"} \xrightarrow{2H_2O,\ O_2} {}^{\ominus}E-CH=O + RCH_2CH=O + NH_3 + H_2O_2 + \text{"flavin"}$$

Studies utilizing deuterium effects were said to favor this second pathway (339).

Smithies and Mann, in connection with enzyme catalyzed formation of heterocyclic compounds, consider that a possibility exists for the production of an aldimine rather than of an aldehyde during the diamine oxidase reaction (331). Both types of primary products could cyclize to form the same heterocyclic products.



Mechanisms leading to the production of aldimines have not been proposed. The isolation of amino-aldehyde derivatives from reaction mixtures and the action of DAOs on monoamino compounds to form isolatable aldehydes tend to make this possibility seem rather remote.

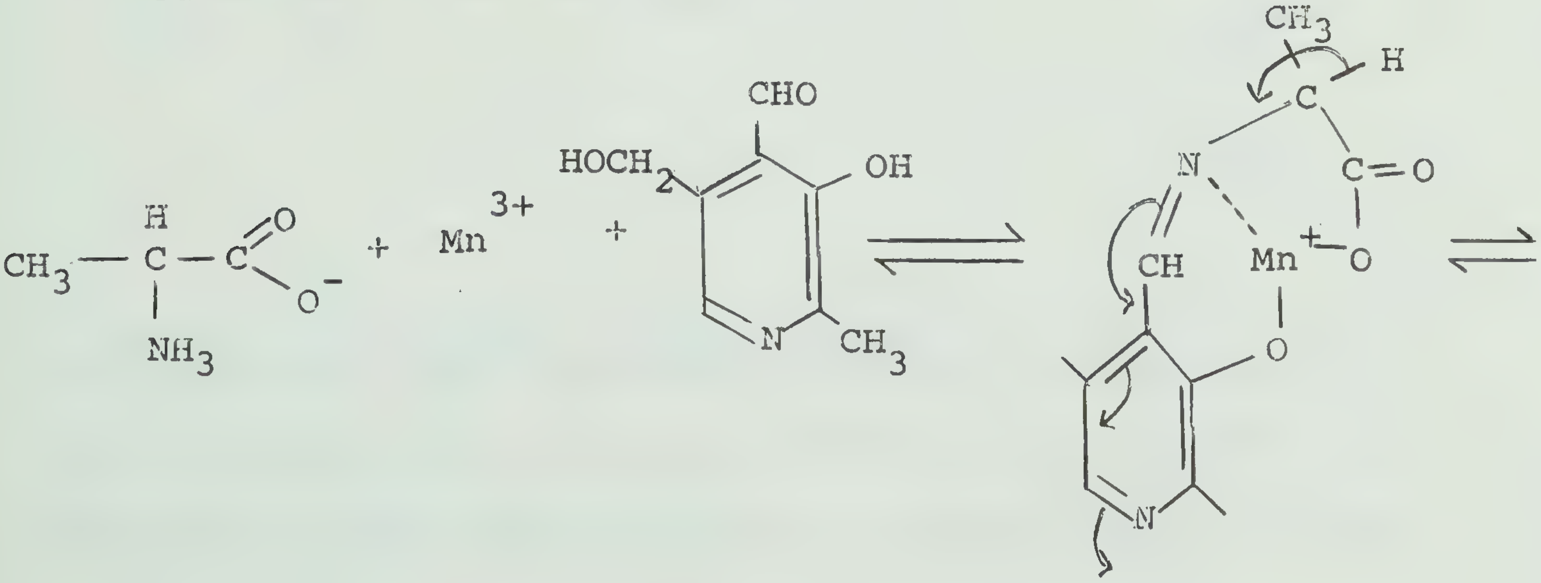
Goryachenkova (433) studied the two schemes proposed by Werle and Pechmann. He tried in two ways to verify the first scheme given above (where the enzyme-amine intermediate is dehydrogenated to an imine that is then hydrolyzed to free ammonia and enzyme). He first tested the possibility of

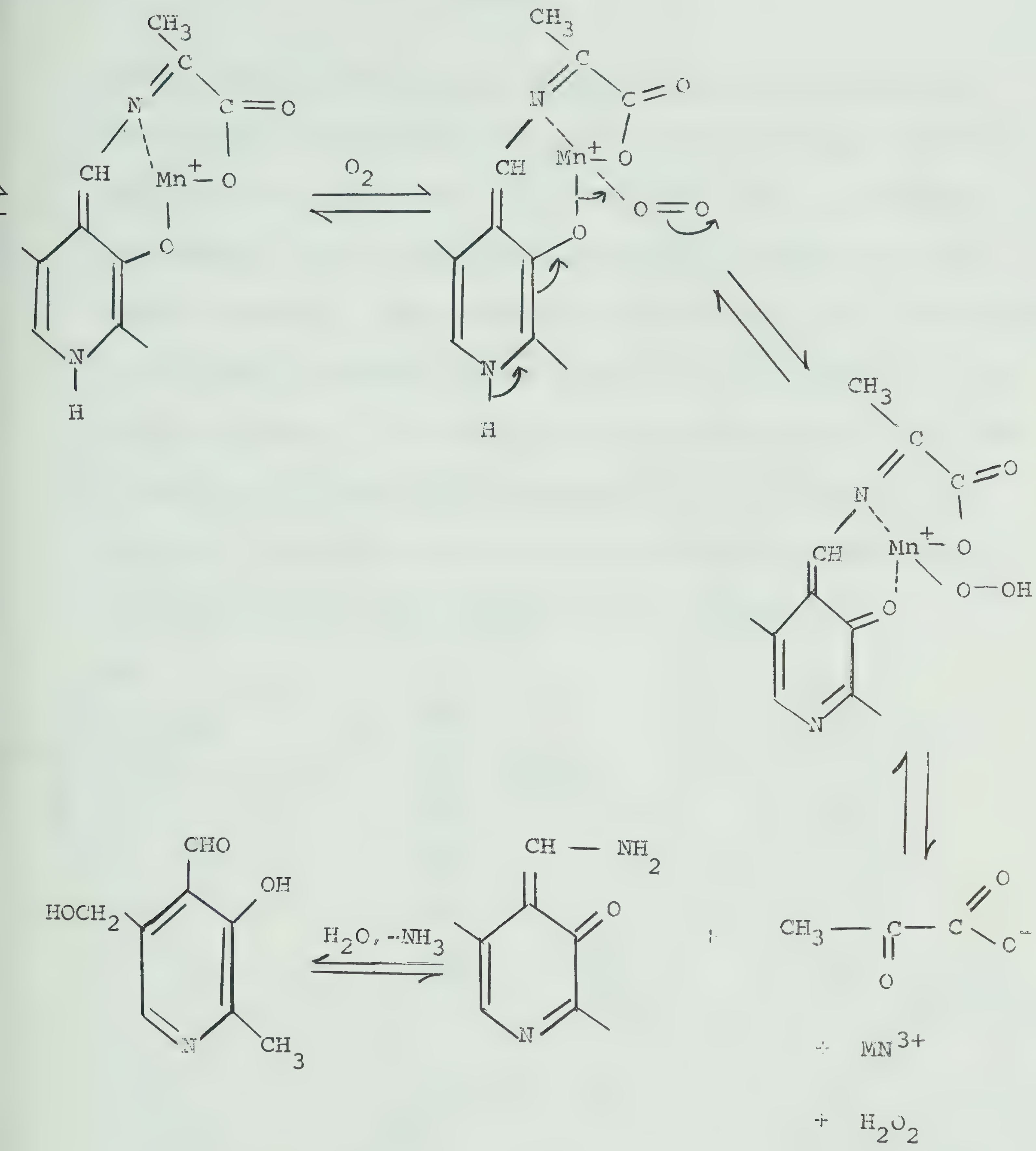
enzymatic transamination (via enzyme-amine) from diamines to pyridoxal, α-keto acids, or to phosphopyridoxal. Secondly, he examined the possibility of enzymatic oxidative deamination of pyridoxamine by DAO. Experimental difficulties and oxidation of the pyridoxamine by DAO made his results difficult to interpret. He succeeded in showing the possible involvement of azomethine-type intermediates but proved or disproved neither of the proposed mechanisms. Kapeller-Adler and MacFarlane also considered these schemes, but again experimental difficulties prevented any conclusions (345).

Hill and Mann (337) consider the reaction in view of pyridoxal-catalyzed model transaminations. They discuss the possibility of a complex of the enzyme protein with a metal-chelate of the Schiff base formed from pyridoxal phosphate and the substrate. The yellow enzyme-substrate complex is considered to be the Schiff base-copper complex. They point out that, as these intermediates are stable under anaerobic conditions, they present great possibilities for the study of the reaction kinetics and mechanism. To date little has been done in this regard. Their mechanistic analogy does not involve flavins and may perhaps be closer than those that require flavins to being correct. Hill and Mann have reported oxygen uptake when pyridoxal phosphate, MnII and amino acids are combined in solutions buffered at pH 7 at 25° (466). This

system both deaminated and decarboxylated the amino acids to produce aldehyde products. The yellow colour of the reaction mixture was thought to be the result of Schiff base formation. They consider that the MnII catalyzes the oxidation of the Schiff bases formed from the pyridoxal and the amino acids. Pyridoxal or pyridoxal phosphate was consumed in the reaction. The best substrates (in decreasing order of effectiveness) were L-lysine, asparagine, β-phenylalanine, L-ornithine and methionine (466). This is a possible model reaction for amine oxidases.

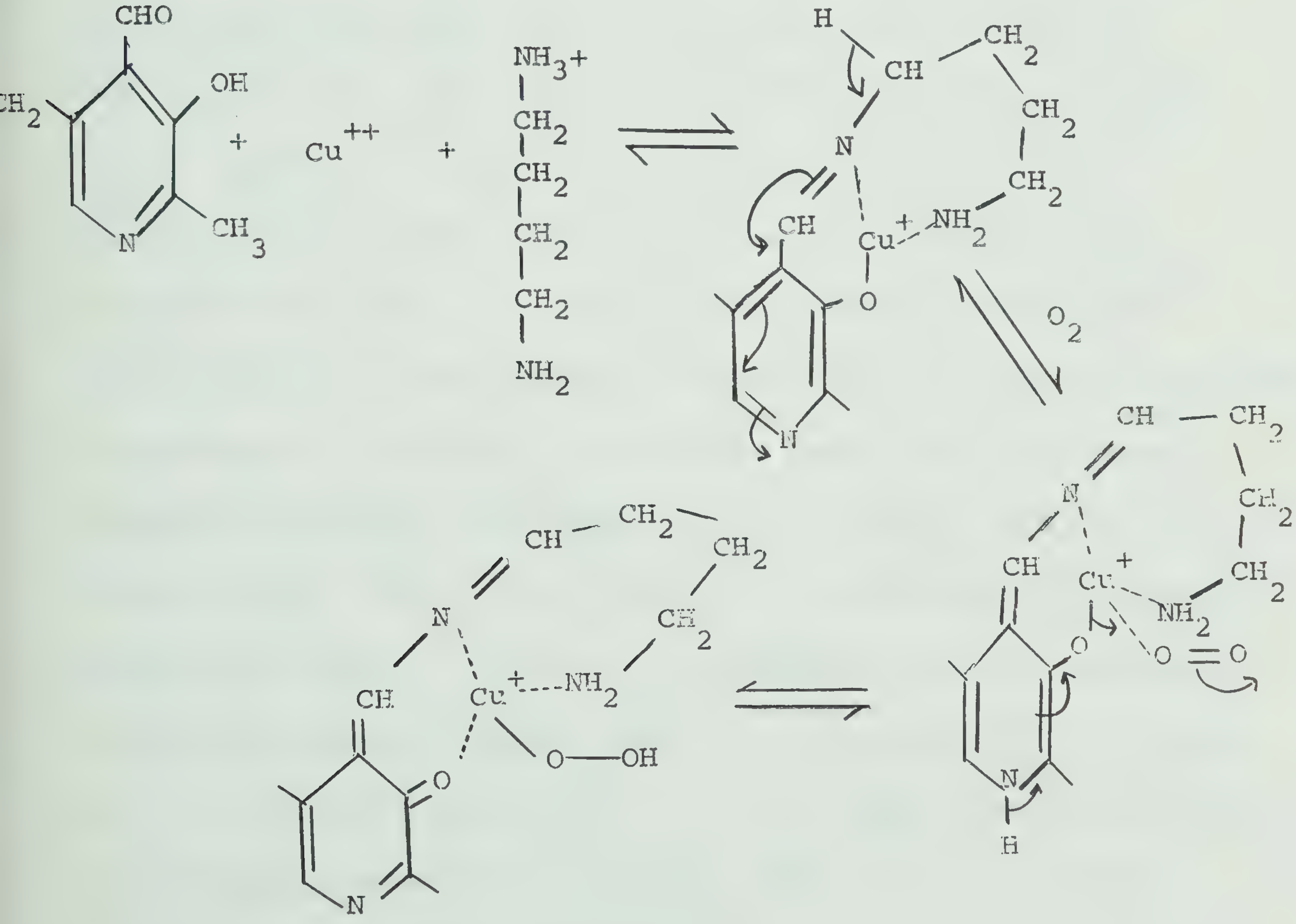
Hamilton and Revesz (1966) have studied similar model reactions among amines (or amino acids), pyridoxal phosphate and metal ions (396). These reactions produce hydrogen peroxide (as shown by the stoichiometry) and products that readily convert into aldehydes. The first two steps in the proposed mechanisms are similar to those proposed for other pyridoxal-catalyzed reactions.

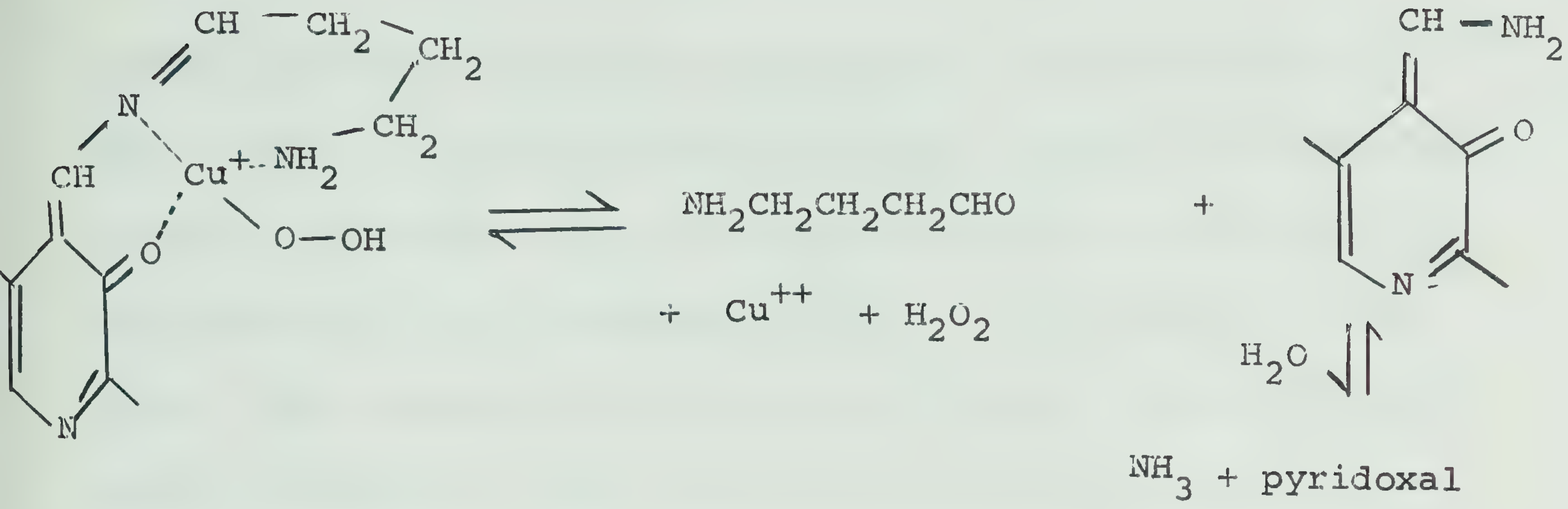




Metal ions are able to transfer electrons by the overlap of their d-orbitals with the p orbitals of the ligand (Hamilton in discussion section of reference 407). In the

model system, amino acids are more readily oxidized than simple amines possibly as a result of the stronger complexes they can form with metal ions (Hamilton, 407). Presumably the protein part of an amine oxidase compensates for this weaker binding. These studies do not explain the disappearance of pyridoxal phosphate noted by Hill and Mann (466) in their similar system. Possibly their more complex system had some other factor present that catalyzed the oxidation of the cofactor. A modification of the Hamilton and Revesz model to involve CuII and putrescine is as follows:





This mechanism is only a model and must therefore differ from the protein-bound enzyme reaction, but it does show how the oxidation could occur with the involvement of a metal ion and pyridoxal. This mechanism does involve one amino group being positively charged and the other acting as a nucleophile, and also shows the possible importance of the chain length in bringing the two groups into the proper orientation. It does not explain the observations on the nature of the chain, nor does it explain the mechanism of the formation of the aldehyde product from the cuprohydroperoxide intermediate. Evidence from ESR has shown that the copper remains cupric and that there is not an amine ligand of the copper (395). This is not reason for discarding the proposed mechanism, however, as,if the intermediates of the reaction were only momentary states (that did not accumulate), a change in the ESR signal would not be detected (Williams and Hamilton in discussion section of reference 407). The involvement of the protein matrix must be considered also in relation to

these questions. The copper is possibly bound directly to the protein - perhaps to a free hydroxyl group or to an amino group (copper-amine complexes are well known). The nature of the binding of the pyridoxal phosphate to the protein is not clear. The strength of the binding indicates that a covalent bond or possibly a very strong non-covalent bond (apolar?) might be involved. Binding through complexed copper would probably be more labile. The binding of copper itself seems exceptionally strong - in some cases there has been no success at all in removing it from the protein. NMR and ESR studies of the model systems could give a great deal of mechanistic information as could careful UV - visible spectrophotometric studies of the enzyme-substrate complex. Information on the copper content of variously degraded DAOs could also be important. There are no reports of any such studies in the literature.

Model systems for transaminations involving only metal ions, pyruvate and amino acids are also known (467). Pyridoxal phosphate is not required in these systems.

(6). Assays for Diamine Oxidase Activity

(a). Detection of diamine oxidase activity

Diamine oxidase activity may be readily detected either by oxygen uptake measurement in the presence of diamines (336, 339, 416), by detection of the hydrogen

peroxide produced (336), by detection of the ammonia liberated (336, 357, 378), or by detection of the reaction product (either the aldehyde form (416) or the cyclic form (419). The degradation of histamine or some other readily detected and determined substrate may be used. These methods all form the bases of the various enzyme assays to be described below. Positive identification of an amine oxidase requires that at least two detection methods be employed and that inhibitors be used to differentiate between DAOs and MAOs. Iproniazid inhibits MAO more than DAO in vitro (436). Semicarbazide and aminoguanidine inhibit DAOs but not MAOs (436). Certain other MAO inhibitors do not affect DAO (441).

(b). Quantitative determination of diamine oxidase activity

There are many methods described in the literature for the determination of DAO activity. These fall into two groups: measurement of substrate disappearance (oxygen uptake, decrease in substrate concentration), and measurement of the appearance of the reaction products (measurement of ammonia, hydrogen peroxide, or of the aldehyde or cyclic condensation product).

(i). measurement of oxygen uptake

This is the oldest and perhaps the most commonly employed method for DAO determination. All work reported in the literature has been with the classical Warburg methods,

but there appears to be no reason why polarographic methods should not be equally applicable. Manometric studies are subject to interference by: (1) other contaminating enzymes that use or produce oxygen or other gases, (2) chemical reactions that involve gas formation or consumption and, (3) reaction of the hydrogen peroxide with peroxidase (a persistant contaminant of plant DAO preparations) or catalase. These sources of error make it imperative that proper controls be used and desirable that some other method be used to confirm the results of the manometric studies (465). Mann (389) observed slow oxygen uptake by DAO reaction mixtures after the stoichiometrically required amount of oxygen had been used. He attributed this to secondary oxidative reactions; similar effects were observed by Cotzias and Greenough (465), who found (with MAO) that certain inhibitors could force the oxygen uptake of their systems to almost follow the expected stoichiometry. Typical results of manometric studies are to be found in references 336, 337, 339, 378, 389, 405, 432. In these studies other methods were used to check the manometric results. Werle and Pechmann found that the rate of clover DAO-catalyzed reactions increased as oxygen tension was increased. Mann did not find this with pea seedling DAO and suggests that oxygen diffusion into the reaction medium was the limiting factor (389). The rates of some monoamine

oxidase-catalyzed reactions have been found to be increased by increasing oxygen tension (468). Warburg manometry has given the bulk of the present information on the behavior of the amine oxidases.

(ii). measurement of substrate disappearance

Methods of determination of DAO activity by following the decrease in substrate concentration are not generally used, with the exception being those DAOs that react with histamine. A method that has had considerable use is a bioassay for histamine based upon its effect on the contraction of guinea pig intestine (469). The method is capable of great sensitivity when properly employed; it is, however, subject to the actions of other, possibly unknown, contaminating materials, and, moreover, lacks appeal as a routine assay method. Werle and Zabel (341) employed this method in their initial survey of plants for histaminase activity, and Kapeller-Adler used it as a check on the results obtained by her microvolumetric method (334, 350). The disappearance of histamine has also been measured fluorometrically (364), and colorimetrically (416, 432). The only other instance of determination of the amount of amine substrate consumed is the use of isotope dilution in the determination of cadaverine excreted in urine after rats were dosed with labelled cadaverine (437).

(iii). measurement of the ammonia produced during the diamine oxidase reaction

Ammonia released may be readily and accurately determined by the various modifications of the Conway microdiffusion technique. A mercuric oxide based method has also been used (331). Exogenous ammonia may interfere and be difficult to exclude and errors also may arise from the diffusion of other, volatile, basic materials - such as the cyclic compounds that form, in some cases, from the primary reaction products (331). Determination of the ammonia is described in references 339, 357, 378, 413, 416, 465 and 470. Ammonia formation and oxygen production may be simultaneously measured with slightly modified Warburg flasks. Nagatsu and Yagi (471) have recently introduced a method for ammonia determination (by an indophenol colour reaction) that does not require prior separation of the ammonia from the reaction mixture and has been used in studies of MAO. Ammonia can also be accurately and readily determined spectrophotometrically utilizing the requirements of glutamate dehydrogenase for ammonia and NADH in glutamic acid synthesis (472). Lorenz, Kusche and Werle (444) have used this method to follow the reactions of plant and animal DAOs. The reaction employed is:

$$\alpha\text{-ketoglutarate} + NH_3 + NADH + H^+ \rightleftharpoons \text{glutamate} + NAD^+ + H_2O$$

and the oxidation of the NADH is followed at 366 mμ.

(iv). measurement of hydrogen peroxide formation

Mondovi, Rotilio, Finazzi, and Scioscia-Santoro measured the hydrogen peroxide produced during the diamine oxidase reaction by its oxidation of hemoglobin (340). Many compounds may be destroyed by coupled oxidation in the DAO reaction and so used as a measure of hydrogen peroxide production. Indigo disulphonate is frequently employed. Kapeller-Adler has developed a method based upon titration of the residual indigo disulphonate with permanganate (334, 350). She finds this "microvolumetric" method accurate and of sensitivity comparable to biological assay methods, but Zeller argues that the method is based upon a complex system of reactions involving DAO as a peroxide generator and some peroxidatic principle. This complexity renders the method sensitive to such interfering factors as CuII ions in the presence of some substrates (329, 473, which alter the reaction rate). Kapeller-Adler seems to have satisfactorily answered her critics (334, 350) and modifications of her method are still in use. Lozzio, Marenzi and Royer (474) determined the residual indigo spectrophotometrically. A similar method was used by Aarsen and Kemp (475) who substituted o-dianisidine for indigo as a hydrogen donor.

(v). measurement of the production of the aldehyde product

The aldehyde produced in the diamine oxidase reaction has been measured through its oxidation by aldehyde oxidase (the NADH formed is measured) (416). Aldehydes react with bisulphite and the residual bisulphite can be determined by iodometric titration. This has been used, but in the case of certain reaction products (especially those from cadaverine and putrescine) iodine uptake occurs regardless of whether or not bisulphite has been added. The uptake of iodine is probably the result of its direct addition to the unsaturated heterocyclic compounds resulting from the condensation of the amino aldehydes produced in the reaction (331, 336). The reactions of these unsaturated heterocyclic compounds with o-aminobenzaldehyde (which may be readily prepared from commercially available o-nitrobenzaldehyde (476)) to form coloured quinazolinium compounds have been used as the basis for a spectrophotometric DAO determination method (419, 425). This method has been used to determine serum amine oxidases (367) and in studies of pea DAO (443). This determination method compared favorably to the manometric methods and is linear over a wide range of enzyme activities. It lacks the sensitivity of histamine bioassays but is said to be equivalent to most of the other chemical methods (419). Lorenz, Kusche, and Werle (444) found this method less dependable than their NADH-dependent ammonia determination.

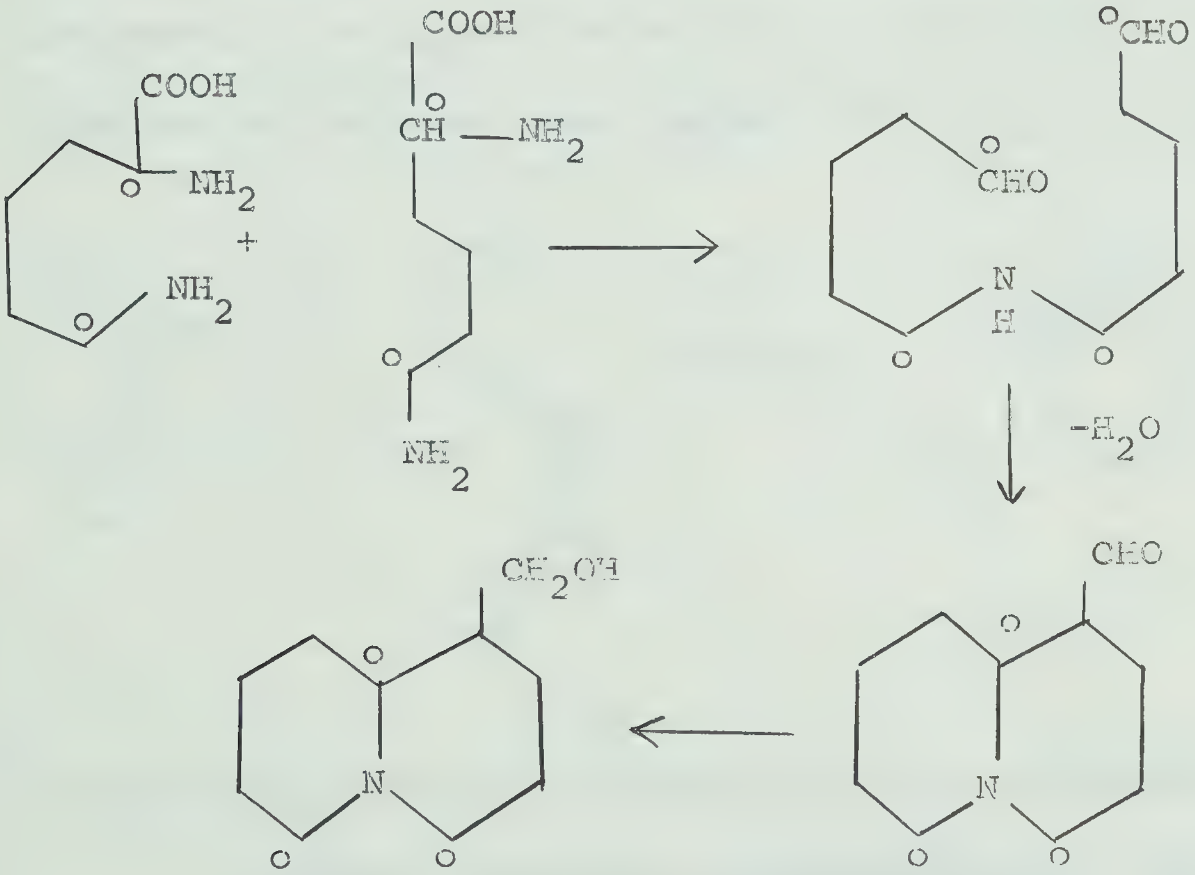
A novel DAO assay procedure is based upon the extractability of the oligomeric products arising spontaneously when cadaverine or putrescine is oxidized. Okuyama and Kobayashi (426) have shown that these products (at pH 8.3) are readily extracted into toluene while the substrate is not appreciably removed. These authors used radioactive substrates and extracted the reaction mixture (after treatment with sodium bicarbonate) directly with a standard liquid scintillation counting medium (a toluene solution containing a primary fluor). Using carefully standardized techniques good reproduceability was obtained as was a linear calibration curve. The sensitivity was a function of the specific activity of the substrate and approached the level of sensitivity obtained with fluorimetric procedures. This method has the advantages of accuracy, specificity, simplicity and sensitivity. These workers found evidence for the formation of mixtures of reaction products, all toluene extractable. This method has been used by its originators in studies of diamine oxidase levels during human pregnancy (477, 478, 478, 480). The method has been modified for determinations in a total volume of 0.1 ml. of reaction mixture (372). A similar technique has been applied in the estimation of MAO activity. The radioactive indole-3-acetaldehyde formed from ^{14}C-tryptamine is extracted with a toluene-based scintillation

counting mixture (481). Less than 0.3% of the substrate was extracted and duplicate runs differed by less than 2%. The assay was not affected by the presence of aldehyde dehydrogenase. Using the same method for DAO determination, Reed (482) reports less than 0.05% of the substrate was extracted when the reaction product was extracted.

F. BIOGENESIS OF THE QUINOLIZIDINE ALKALOIDS

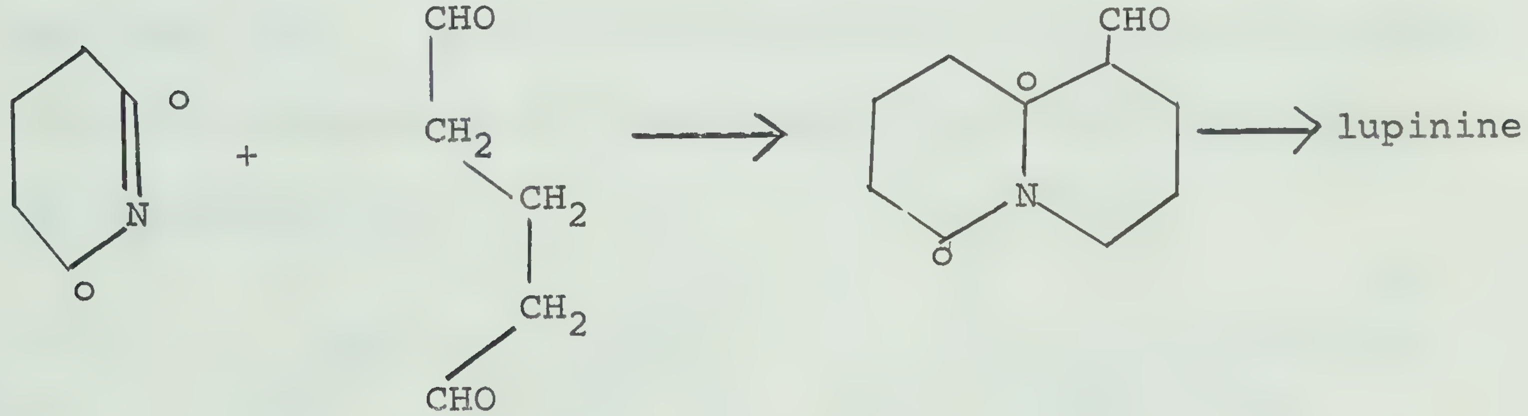
(1). Theories Proposed on the Bases of Chemical Considerations

Several ideas for the biological origin of quinolizidine compounds have been proposed by chemists who have based their speculations upon structural similarities and known chemical reactions. In 1931, Schöpf proposed that two molecules of lysine could give rise to lupinine via an iminodialdehyde (34, 485) and Mannich cyclizations (cf. diagram). Open circles have been added represent the positions of carbon-14 atoms, assuming that a symmetrical intermediate is interposed between lysine-2-^{14}C and the other intermediates and products.



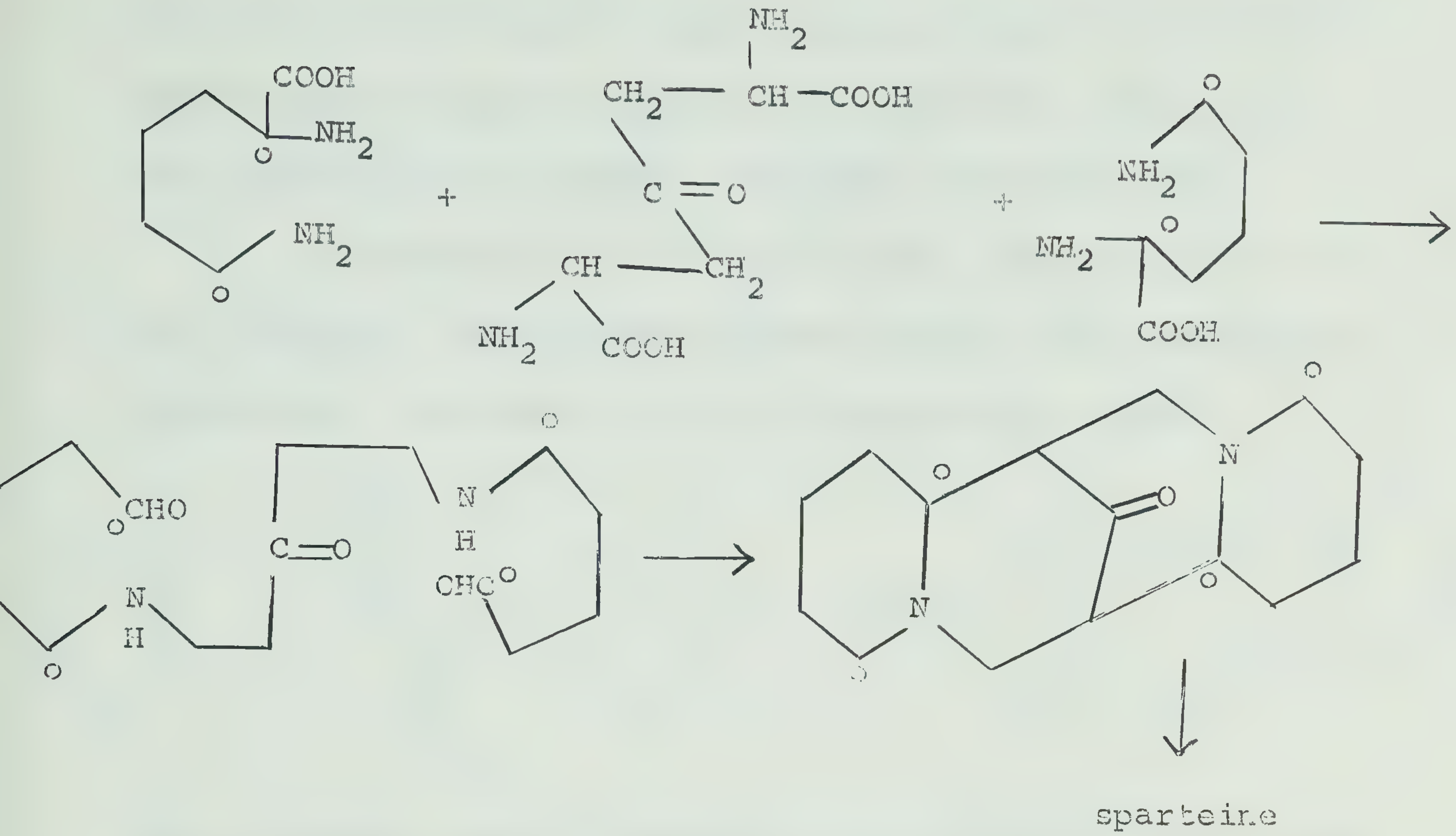
This proposal was also made by Sir Robert Robinson (34, 486). The iminodialdehyde, when formed in chemical reactions, spontaneously cyclizes to the intermediate lupinine-aldehyde, the reduction of which affords racemic epilupinine (486, 487, 497). Similar chemical syntheses (from substituted and oxidized dialdehyde intermediates) have yielded the less stable isomer, racemic lupinine (486, 497). Other workers have used analogous methods to synthesize some of the closely related pyrrolizidine alkaloids, again confirming the possibilities of Robinson-Schöpf biogenetic hypotheses (488).

Schöpf also proposed that lupinine might arise from the reaction of glutardialdehyde (or a derivative) and Δ'-piperideine (arising from lysine) (34, 485, 489, 492).

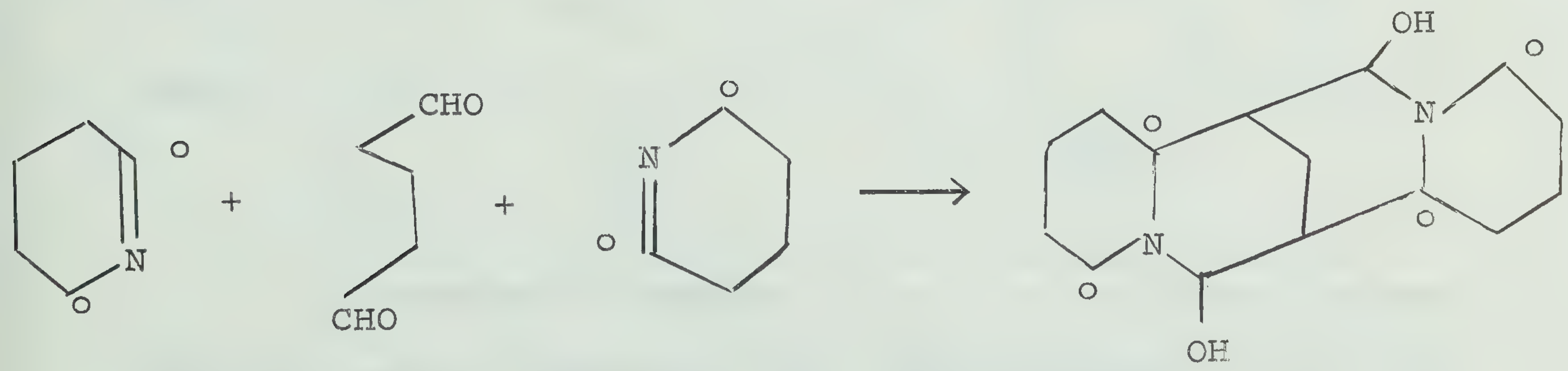


Tracer studies tend to confirm the former hypothesis (490).

Robinson proposed that sparteine and other tetracyclic alkaloids could arise from two molecules of lysine and one molecule of γ-keto-α,α'-diaminopimelic acid (27, 34).



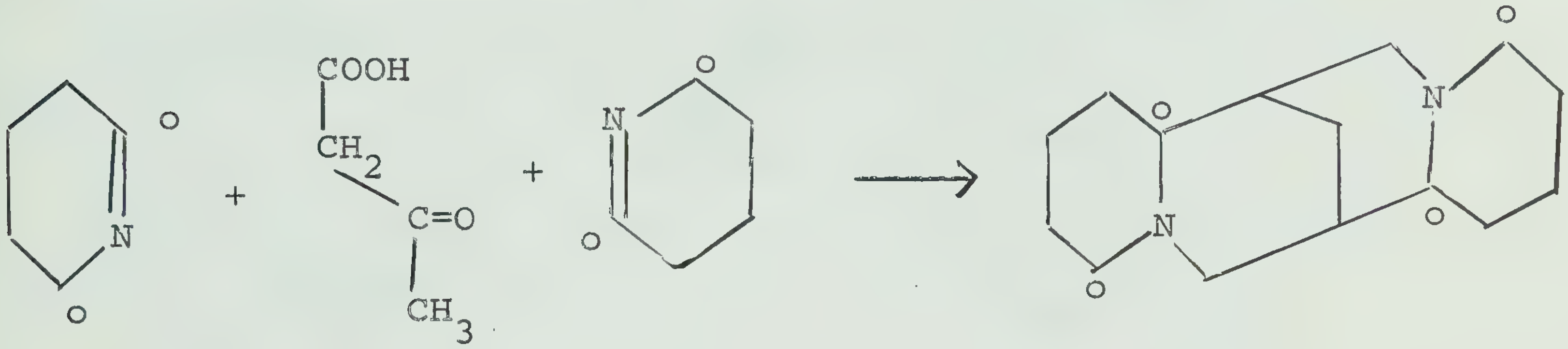
Wenkert has argued that dicarboxyacetone or ketodiaminopimelate are unrealistic proposals and are unlikely to enter into these reactions (491). He proposes that glutaraldehyde could condense with two molecules of Δ'-piperideine (from lysine) to give dihydroxysparteine.



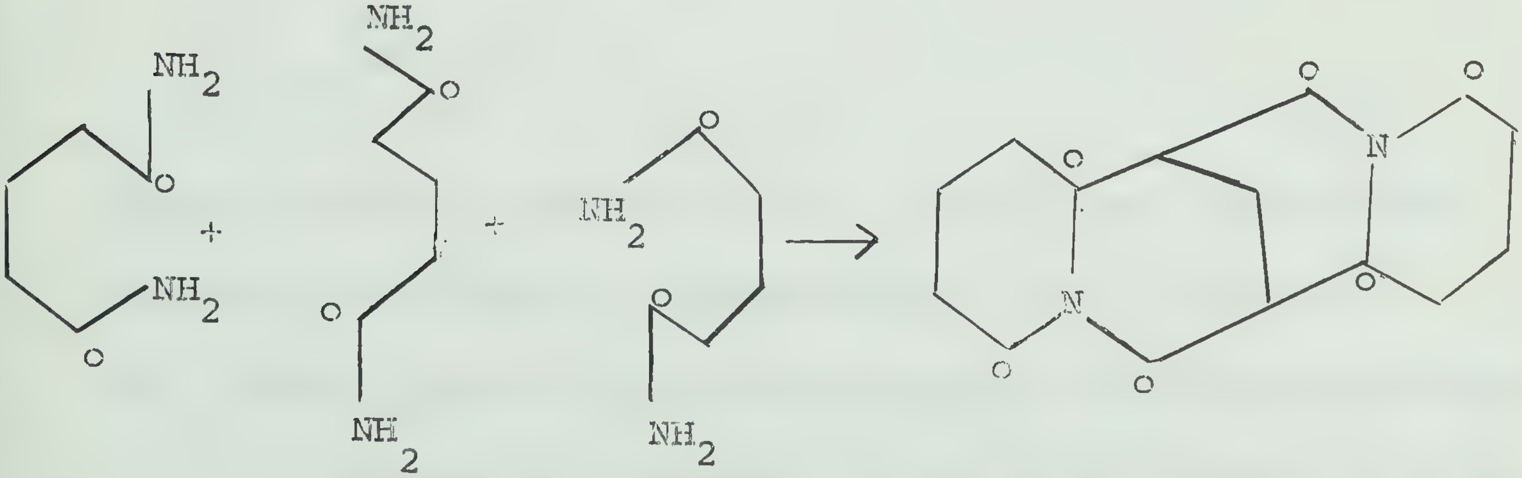
Attempts were made to carry out the Robinson suggestion using formaldehyde, δ-aminovaleraldehyde (a biological equivalent of lysine produced *in situ* by slow

hydrolysis of its acetal) and acetonedicarboxylic acid (495). Other workers failed to confirm this synthesis (492), but later studies using changed conditions succeeded (34).

Van Tamelen and Foltz proposed, and carried out in the laboratory, a slightly different scheme that involved △'-piperideine, acetoacetic acid and formaldehyde (493).

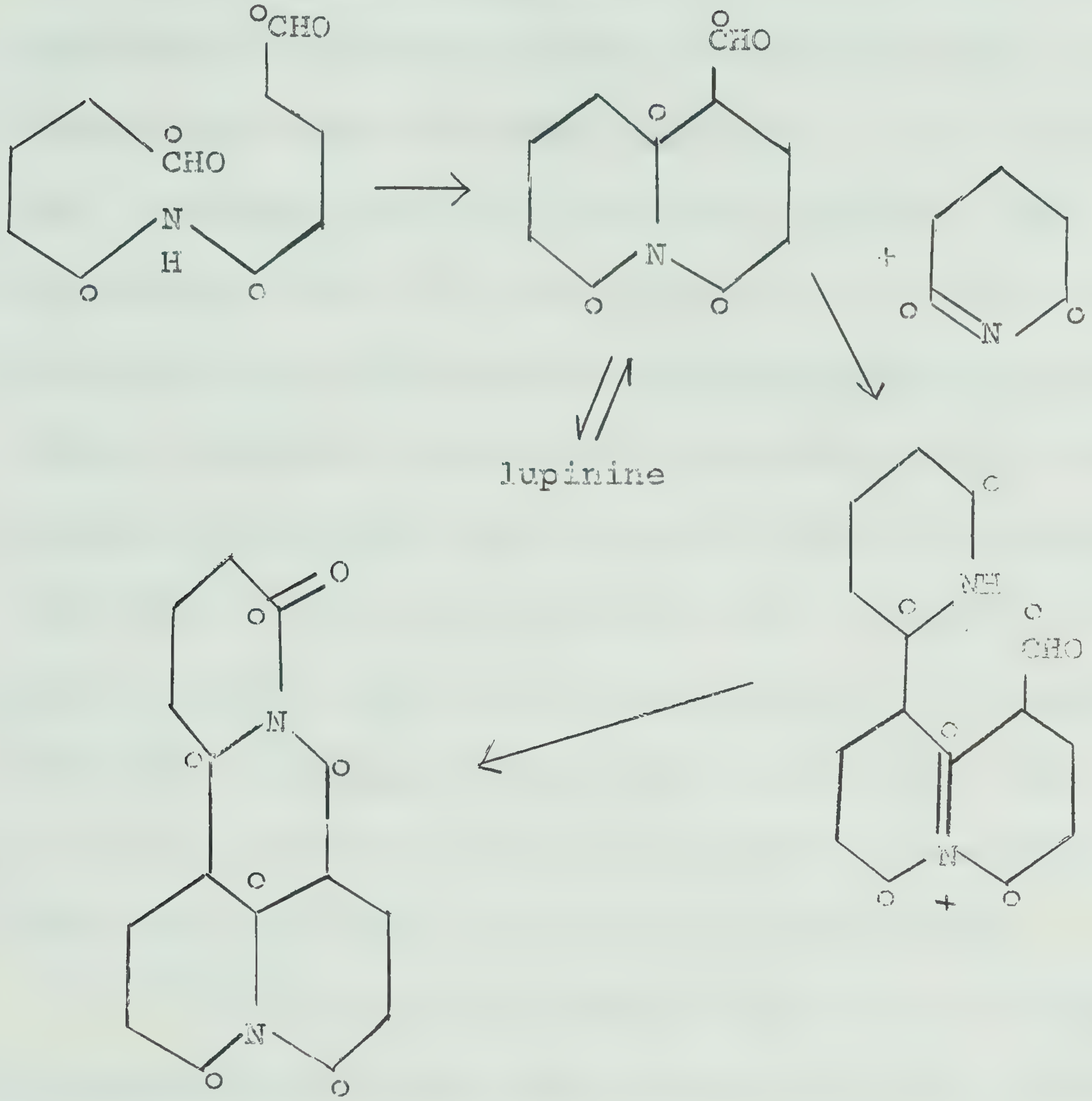


Tracer evidence tends to suggest that sparteine arises solely from three molecules of lysine *via* a symmetrical intermediate giving a probable distribution of label as follows (34):



Biosynthetic schemes for matrine have been proposed involving tetrahydroanabasine and glutardialdehyde (494) and the reaction of △'-piperideine with lupinine-aldehyde (490). A chemical synthesis similar to the former has been reported

(496). The latter proposal, however, is more in keeping with evidence from tracer studies.



Racemic products result from all of the above schemes that have been successfully demonstrated in the laboratory.

(2). Tracer Evidence in Studies of Quinolizidine Biosynthesis

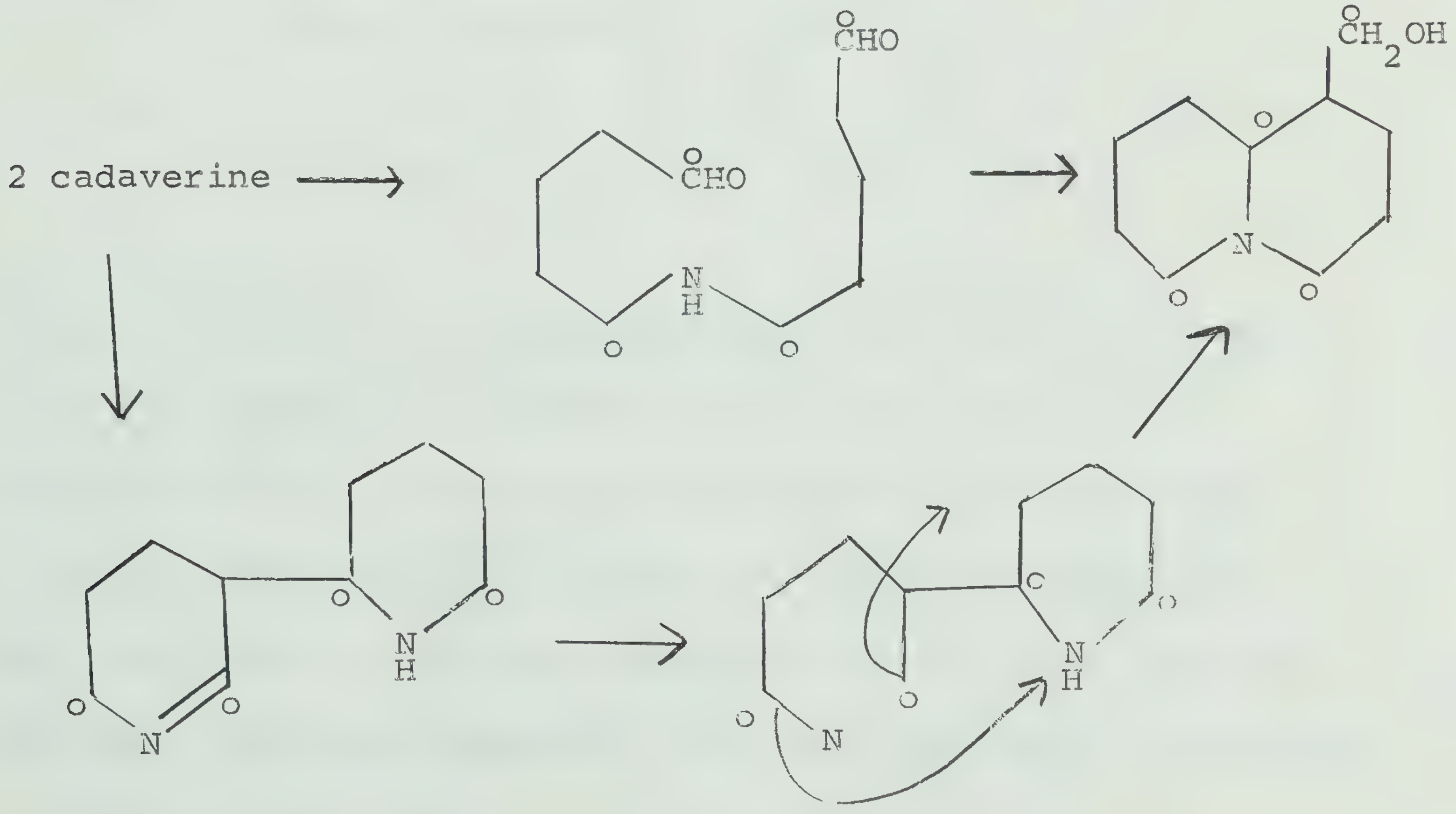
Lysine is generally favored as the precursor of at least the major part of the carbon skeleton of the quinolizidine alkaloids. Section D6 (p. 92 above) reports on some of the studies on the levels of free and protein bound amino acids in bitter and sweet lupines. Such studies have contributed

relatively little to knowledge of the precursors of quinolizidine alkaloids. Arginine has also been proposed as a quinolizidine precursor on the basis of amino acid level studies (298). Tracer studies with arginine tended to disprove this hypothesis (250, 509). A recent study of lysine and arginine levels, their biosynthesis from $^{14}CO_2$, and alkaloid biosynthesis from $^{14}CO_2$ in sweet and bitter varieties of Lupinus angustifolius failed to reveal significant differences in levels of free lysine and arginine or in their specific activities or in the specific activities of the alkaloids (498, 499). Evidence does not appear to favor arginine as a precursor of the quinolizidine alkaloids, although the hypothesis has not yet been shown to be false. There has been no suggestion of a mechanism through which arginine might enter the alkaloid skeleton.

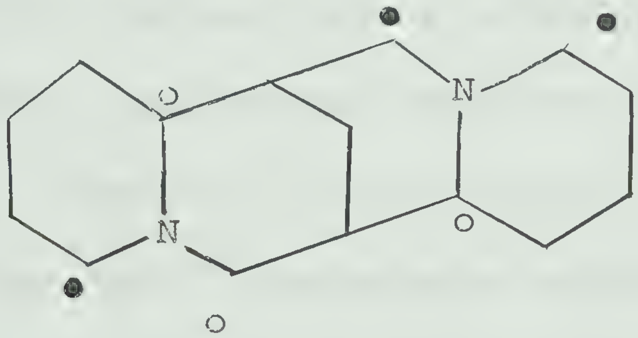
The bulk of the tracer studies on the biosynthesis of these alkaloids has been carried out by H.R. Schütte who has reviewed this work in references 500, 501 (1965, 1966 - in German) and 34 (1963 - in English). In 1959 Schütte and Nowacki fed cadaverine-(1,5)-^{14}C and lysine-2-^{14}C to 9 week old plants of Lupinus luteus and observed incorporations of 4.5 to 5.3% and 1.25 to 3.5% respectively into lupinine (502). Incorporations into sparteine were said to be higher. Jaminet fed lysine-U-^{14}C to plants of Cytisus scoparius and isolated labeled cadaverine, piperideine (presumably as a derivative), 1,2,3,6-tetrahydro-

pyridine-2-carboxylate and a presumed sparteine precursor (503). Yellow and white lupine plants exposed to $^{14}CO_2$ had 0.19% of the total incorporated radioactivity present in the alkaloid fraction (504).

Glutamic acid was shown to be a poor precursor of quinolizidine alkaloids in *L. luteus* and when lysine-2-^{14}C or cadaverine-(1,5)-^{14}C was fed one quarter of the radioactivity of the isolated lupinine was found in the $-CH_2OH$ group (C11) (505). This was thought to favor a biosynthesis from two molecules of cadaverine. Such a label pattern could arise from an iminodialdehyde intermediate or from a rearrangement of tetrahydroanabasine (505).



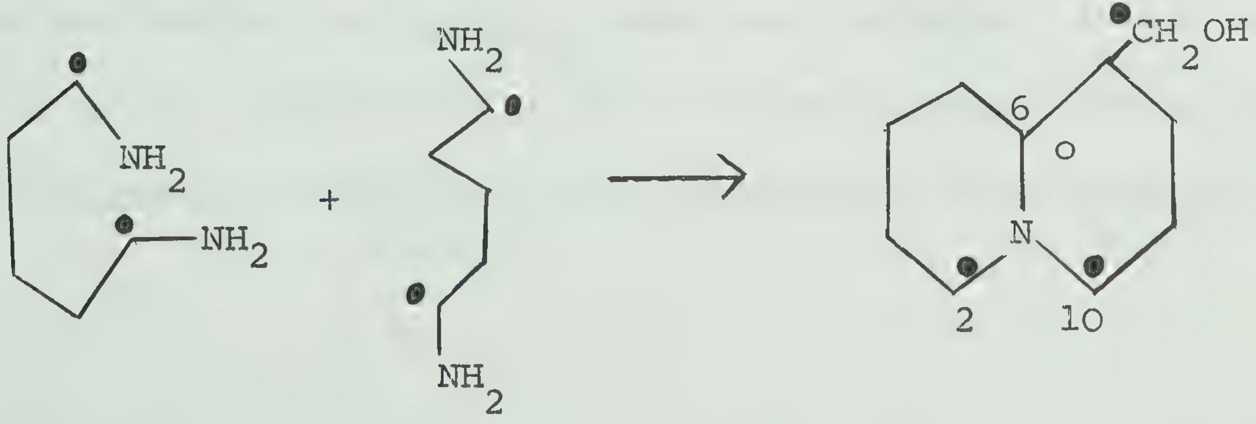
Further studies with lupines fed $^{14}CO_2$ indicated that the green parts of the plants were primarily involved in alkaloid biosynthesis (282). In such experiments, the specific activity of lupinine was very much greater than that of sparteine. These workers checked for carbon-14 isotope exchange reactions and found none. Plants of L. luteus fed cadaverine-(1,5)-^{14}C formed radioactive sparteine that was partially degraded to locate the positions of the radioactive atoms (506). The following diagram shows the positions where activity was found (●), the known inactive atoms, and those places suspected (but not shown) of being positions of ^{14}C (o).



Similar studies with L. angustifolius indicated that lupanine, as well as sparteine, originated from three molecules of cadaverine (507). Mothes reported studies that showed that L. luteus shoots rapidly incorporated lysine and cadaverine into the alkaloids, but that incorporation was very slow from glutamic, formic and pipecolic acids (all proposed as precursors at various times) (508).

Further degradation studies were reported in 1962 that showed lupanine and hydroxylupanine in L. angustifolius were derived from three molecules of cadaverine and had label distributions similar to that shown above (510).

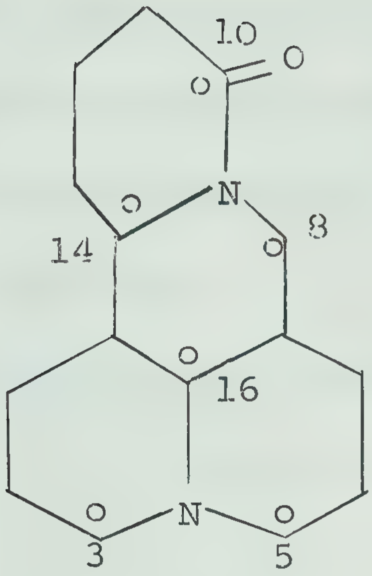
Other workers fed labeled lysine, pipecolic acid, δ-aminovaleric acid, ornithine, and formic acid to L. angustifolius shoots for 48 hours (512). Their results favored a biogenesis of lupanine from three molecules of lysine, possibly with cadaverine or pipecolic acid on the pathway. Neither ornithine, δ-aminovaleric acid nor formate were good precursors of lupanine. Cadaverine fed to Sophora tetraptera labelled matrine in a pattern again indicating that this tetracyclic alkaloid originated from three molecules of cadaverine (511). Further degradations of lupinine isolated from yellow lupines fed ^{14}C-cadaverine showed that C2 and C10 each contained one quarter of the total activity of the molecule, thus confirming its origin from two molecules of cadaverine (513).



It is presumed that the remaining quarter of the activity resides at C6.

Mironenko and Zaben'kova fed lupine plants glycine-2-^{14}C, D-glucose-U-^{14}C, lysine-1-^{14}C, and tyrosine-1-^{14}C and observed that compounds similar to glycine were the best utilized alkaloid precursors (514). These workers report incorporations of label into the alkaloids from all substrates tried. Incorporations from lysine-1-^{14}C and tyrosine-1-^{14}C seem particularly hard to explain. Reference 523 quotes later work of Mironenko that reported no incorporation of label from lysine-1-^{14}C into quinolizidine alkaloids.

Schütte reported studies in 1963 that showed formate and pipecolic acid to be very poor precursors of lupine alkaloids, glutamic acid to be a poor precursor, and lysine and cadaverine to be very good precursors (515). Shibata and Sankawa fed DL-lysine-2-^{14}C, cadaverine-(1,5)-^{14}C, and piperideine-6-^{14}C (presumably this would be Δ'-piperideine-2,6-^{14}C and would be obtained through its equilibrium with a tripiperideine) to Sophora plants and isolated labeled matrine after 3 to 6 weeks (516). Degradation of the alkaloid revealed the following labeling pattern originating from lysine-2-^{14}C.



C10 activity=C8=C3=C5=C14=C16

This appears to confirm that matrine arises from three molecules of lysine through a symmetrical intermediate. Labeled piperideine was a less effective precursor than was lysine or cadaverine and gave 10% of the total incorporated activity at C10 and 90% at C3 and C5 (516). It was suggested that the C10 - containing part of the molecule was derived from tetrahydroanabasine (in equilibrium with △'-piperideine) and that the C3 and C5 parts were derived directly from △'-piperideine. As this species exists only in oligomeric forms (417) they must have fed a material similar to a tripiperideine that is in equilibrium with the monomer in solution. Results based upon the feeding of such highly reactive compounds must be reviewed with suspicion.

Nowacki and Byerrum studied the incorporation of various proposed precursors into lupanine in shoots of L. angustifolius (155). The following table (after 155)

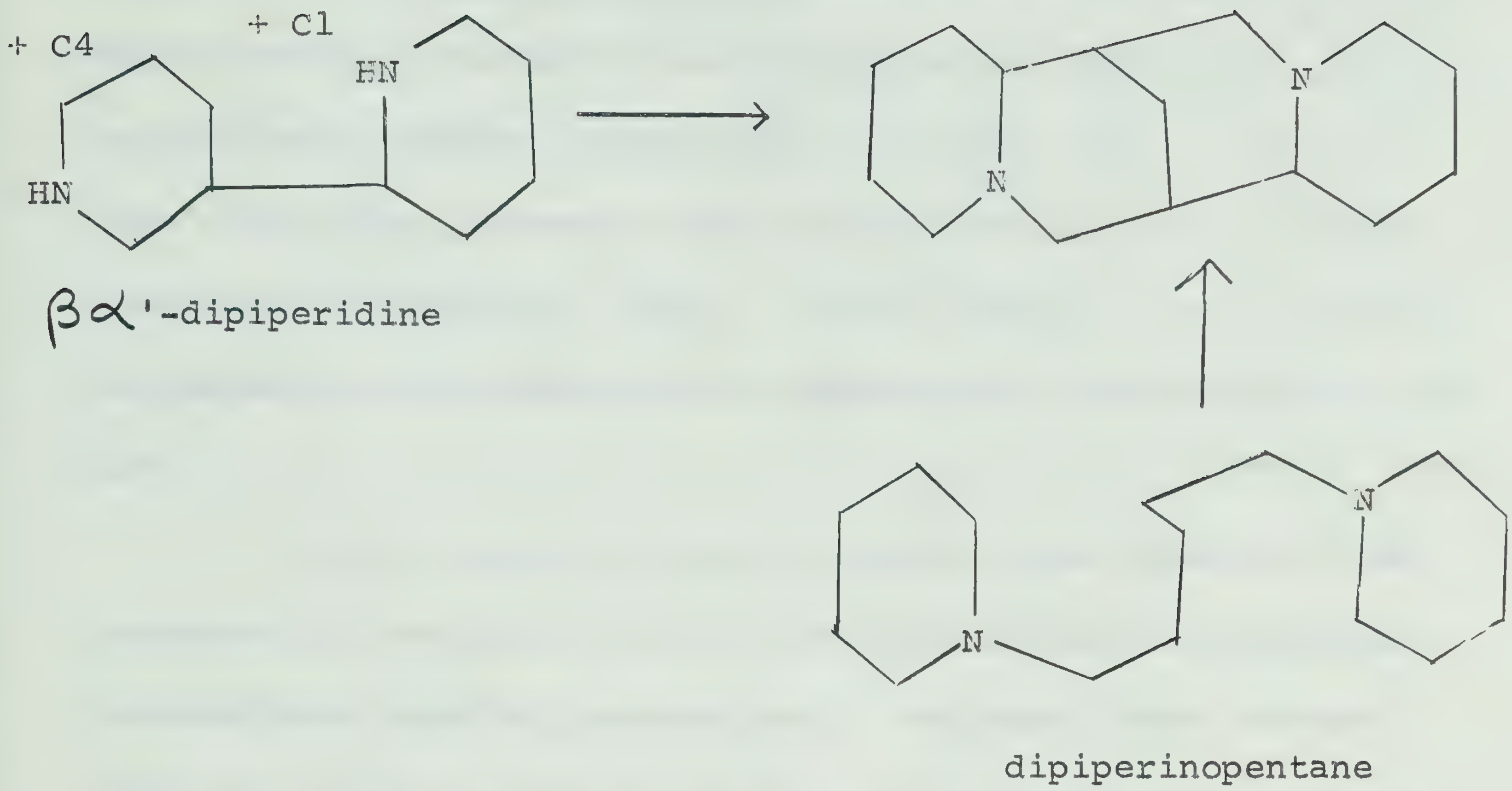
summarizes their results. (Dilution was obtained by dividing the specific activity of the precursor by the specific activity of the isolated lupanine).

substrate	dilution
cadaverine-(1,5)-^{14}C	21
lysine-2-^{14}C	55
succinic acid-(2,3)-^{14}C	83
acetate-2-^{14}C	159
acetate-1-^{14}C	198
aspartic acid-3-^{14}C	275
γ-aminobutyric-1-^{14}C	999
pipecolic acid-2-^{14}C	1829
pipecolic acid-^{3}H (random)	1930
δ-aminovaleric acid-1-^{14}C	9998
ornithine-2-^{14}C	12111
formate-^{14}C	18270

It is apparent that cadaverine, followed by lysine, is the best precursor of lupanine. Degradation of the lupanine formed from these sources gave the by now familiar labeling pattern indicating that three molecules of cadaverine were converted to the alkaloid. These authors show how lysine could arise from aspartate and from some of the other precursors fed. Their evidence from the degradation of lupanine derived from plants fed these various precursors seems to confirm their speculations. The lysine-cadaverine theory received further support from studies of sparteine biosynthesis in Sarothamnus scoparius and L. luteus (517). When lysine-$[2\text{-}^{14}C,\ \alpha\text{-}^{15}N]$ was fed sparteine containing ^{15}N was isolated. The $^{14}C/^{15}N$ ratio indicated that lysine entered sparteine through a

symmetrical intermediate such as cadaverine. Cadaverine was shown to be a precursor of sparteine in *Chelidonium majus* (518). Schütte and Hindorf repeated their studies on lupinine biosynthesis in *L. luteus* and again found that it forms from two molecules of lysine through cadaverine (519). The distribution of label after feeding of cadaverine-(1,5)-^{14}C and lysine-2-^{14}C indicated that cytisine and N-methylcytisine in *Cytisus laburnum* are formed by a biological degradation of the sparteine skeleton formed from three molecules of cadaverine (520, 521). The possibility that tetrahydroanabasine might be involved in lupinine biosynthesis was tested by Schütte, Sandke, and Lehfeldt who fed 2,6, 2',6'-^{14}C-tetrahydroanabasine to *L. luteus* plants (522). Although lysine and cadaverine were rapidly incorporated into lupinine, tetrahydroanabasine was incorporated only to a very slight extent.

Nowacki, Nowacka and Byerrum fed plants of *L. angustifolius* lysine-2-^{14}C, $\beta\alpha'$-dipiperidine, dipiperidinopentane-(1,5)-^{14}C, and N-methylcytisine methyl-^{14}C (523). Only lysine gave a significant amount of radioactivity in isolated lupanine and so it was concluded that these intermediates were probably not precursors of the tetracyclic quinolizidine alkaloids.



These workers failed to find evidence for decarboxylation of lysine in *L. angustifolius* and also failed to find cadaverine, but stressed that other evidence strongly favors lysine as a precursor with a symmetrical intermediate being involved (523). Schütte and Hindorf found that the label patterns of lupanine and hydroxylupanine in *L. angustifolius* were the same if either lysine-2-^{14}C or cadaverine-(1,5)-^{14}C were fed (524). They confirmed the work of the Nowackis and Byerrum in finding dipiperinopentane not to be an intermediate. The biosynthesis of matrine from three cadaverine units was again confirmed in 1965 (525).

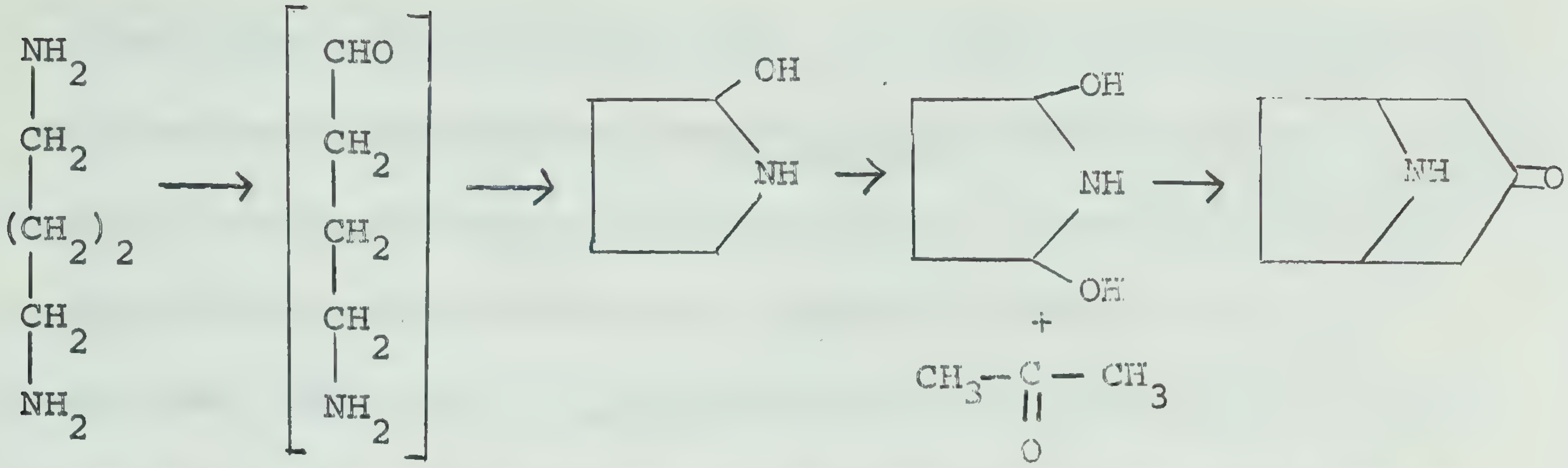
In 1966, further studies on the biosynthesis of cytisine and N-methylcytisine were reported. Pöhm found that

young plants of Cytisus laburnum were able to methylate cytisine (which is fairly toxic to the plants) to the less toxic, more metabolically stable N-methyl alkaloid (526, 527). There is some preliminary evidence that N-methylcytisine can be formed from N-methylcadaverine (528). Current ideas on the conversion of lysine to quinolizidines are summarized in references 501 and 529.

In all types of plants studied lysine appears to be converted to the quinolizidine alkaloids through a symmetrical intermediate (possibly cadaverine). The exact steps between lysine and the alkaloids are still unknown and remain a matter for conjecture and further experimentation.

(3). Possible Role of Diamine Oxidase in Quinolizidine Alkaloid Biogenesis and in that of Related Alkaloids

In 1943 B.T. Cromwell suggested that putrescine was a possible precursor of hyoscyamine in Atropa and Belladona (356). He was able to prepare a plant extract that formed ammonia and an aldehyde with consumption of oxygen when putrescine was the substrate. He proposed that an aminoaldehyde, formed from putrescine, lead to hyoscyamine through tropine. The presence of traces of putrescine and N-methyl putrescine in the leaves at certain stages of growth was demonstrated.



These studies were the first to indicate that possibly diamine oxidase was involved in the biosynthesis of certain alkaloids.

Studies with model systems (see above - section F1) indicated that sparteine and other quinolizidine alkaloids could be derived from aminoaldehydes that in turn could be obtained through the action of diamine oxidase on cadaverine.

In 1952, Blashko discussed the possible role of amine oxidases in alkaloid biosynthesis (530). Mann and Smithies were among the first to study the possible role of DAO in alkaloid biosynthesis (331). They were able to show that the DAO-catalyzed oxidation of putrescine, cadaverine and L- and D-lysine produced Δ^1-pyrrolines, Δ^1-piperideines, and 2,3,4,5-tetrahydropicolinic acid respectively and that these products could readily be hydrogenated. They proposed that these DAO reaction products were possible first stages in the biosynthesis of various alkaloids containing these ring systems (331). Hasse and Maisack also studied the oxidation of putrescine and cadaverine by plant DAO (420). Tetrahydroanabasine was obtained from cadaverine and was oxidized to anabasine by other factors

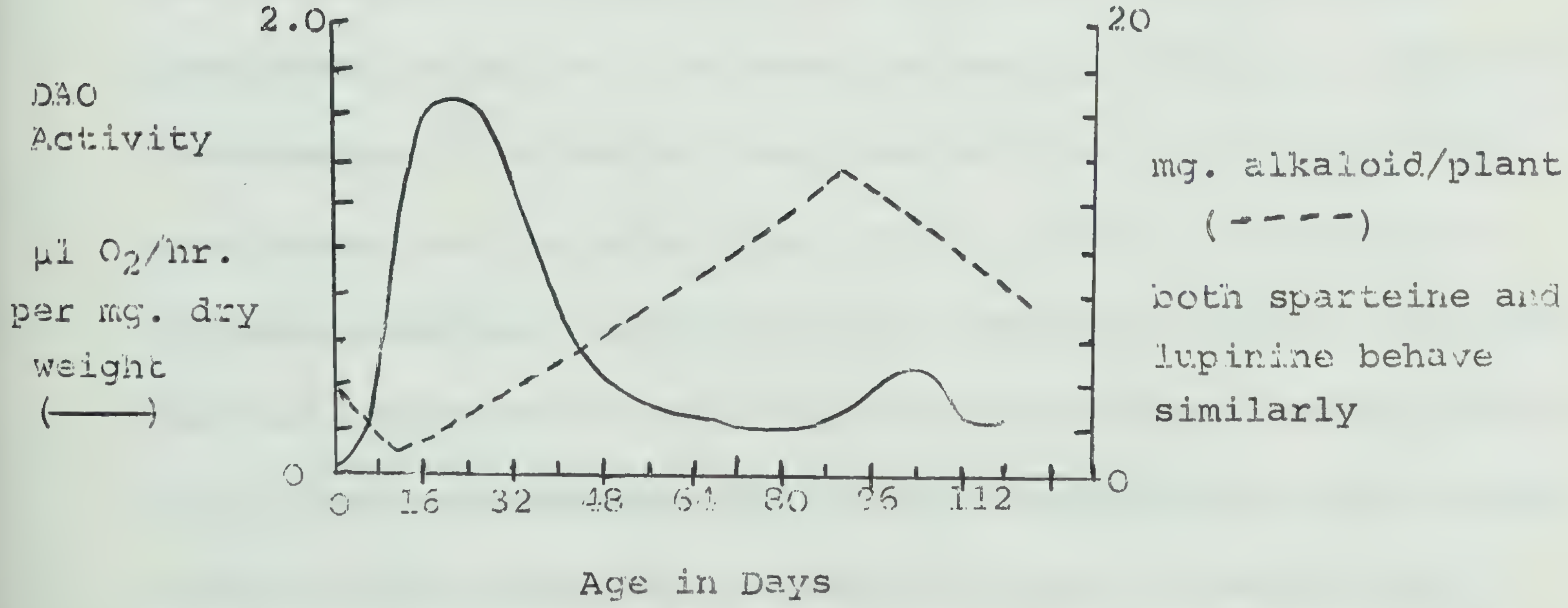
in the plant extracts (420, 421). Tetrahydroanabasine has been proposed as a precursor of the *Nicotiana* alkaloid anabasine (331) and of the quinolizidine alkaloids (522). The presence of Δ'-piperideine compounds from DAO oxidized cadaverine has been confirmed (358, 531). Δ'-piperideine has been proposed to be important in the biogenesis of lupine alkaloids.

Suzuki and Nammo, in 1959, were able to show that a crude extract from *L. luteus* seedlings was able to oxidize both cadaverine and L-lysine, and this oxidation was shown to be catalyzed by a diamine oxidase (377). The authors consider this particularly interesting in view of the possible relationship between lysine and the quinolizidine alkaloids. Suzuki then examined seedlings of *L. luteus* for L-lysine decarboxylase activity (374). He was unable to find any lysine decarboxylase activity, although the tissues had the ability to readily oxidize exogenous cadaverine. Suzuki later partially purified a diamine oxidase from *L. luteus* (357).

A review (in Russian) of work done up to 1961 on the role of DAO in alkaloid biogenesis is available (532). Hasse, Homann, and Schührer found that pea DAO formed Δ'-piperideine-6-carboxylic acid from lysine (427). The 2-carboxyl compound was not formed. An enzyme from Neurospora (ω-hydroxy-α-amino acid dehydrogenase) catalyzed a pyridine nucleotide dependent oxidation of α-amino-ϵ-hydroxy caproic acid (hexahomoserine)

to Δ'-piperideine-6-COOH (427). Δ'-piperideine-6-carboxylic acid was shown to be formed from lysine in yeasts and fungi (428).

Cromwell and Roberts proposed that γ-coniceine was formed from lysine in hemlock through a Δ'-piperideine intermediate but were unable to find any DAO activity (355). Schütte, Hindorf, Mothes and Hübner considered the possibilities that DAO was involved in the formation of quinolizidine alkaloids through either an oxidation of cadaverine or of lysine (517). A more detailed study of diamine oxidase in quinolizidine biosynthesis was carried out by Schütte, Sandke, and Lehfeldt (522). They studied the levels of diamine oxidase in L. luteus and found that diamine oxidase activity was low when alkaloid accumulation was highest (see figure below, after 522).



This behavior does not appear to favor the involvement of DAO in quinolizidine biosynthesis and might indicate that high

diamine oxidase activity could be involved in reactions using up alkaloid precursors. Labeled tetrahydroanabasine was not converted appreciably to quinolizidine alkaloids by *L. luteus* (522).

Lupinus angustifolius plants fed lysine-2-^{14}C were able to incorporate it into lupanine, but no labeled cadaverine could be isolated (523). Unfortunately, these workers did not add unlabeled cadaverine until after the tissue was killed and it is thus possible (although not too likely) that all of the lysine fed was metabolized or decarboxylated and the cadaverine resulting oxidized before the killing of the tissue. No compounds similar to tetrahydroanabasine were found in lupine plant extracts although such compounds were commonly present in closely related genera. The apparent absense of cadaverine, and especially of lysine decarboxylase in lupine plants is perplexing as all of the tracer evidence points to a symmetrical intermediate involved in the incorporation of lysine-2-^{14}C into quinolizidine alkaloids. It is possible that lysine is directly oxidized by DAO to piperideine-6-carboxylic acid that is then converted to Δ'-piperideine (perhaps through pipecolic acid as an intermediate) (523). Δ'-piperideine should be an equally good symmetrical intermediate and, if enzyme bound, might not undergo polymerization reactions. Any carbon-14 isotope effects leading to asymmetry in Δ'-piperideine from

lysine-2-^{14}C would probably be less than the experimental error. Studies with lysine labeled both in the methylene chain and in the carboxyl group should be of great interest. ^{15}N labeling might resolve the question as to whether the ϵ-NH_2 is lost before or after the carboxyl group.

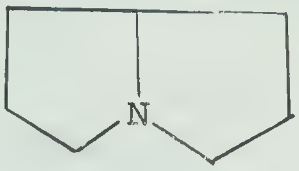
Chemical oxidation of lysine (by NaOCl) has been shown to result in Δ'-piperideine and its derivatives (533). L-Amino acid oxidase (from mussels) produced a product (2-piperidone) quite different from that resulting from DAO oxidation of lysine (534).

Schütte, Knöfel, and Heyer isolated and partially purified diamine oxidase from L. luteus (391). They found diamine oxidase activity to be maximal in plants 12 to 14 days old after which the activity level remained constant and fairly high. This is in contrast to earlier results (522) that indicated a maximum in DAO activity at 12 to 16 days followed by a drop to low levels of activity. DAO activity was highest in the seedling root and shoot, while none was detected in the cotyledon. The purified enzyme oxidized DL-lysine, cadaverine and putrescine, but apparently failed to oxidize L-lysine (391). Hasse has discussed the various enzymes that may possibly be important in forming quinolizidine alkaloids from lysine (404). He considers other enzymes that may act on lysine rather than DAO and feels that if DAO is involved it

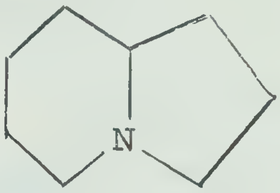
might act upon lysine directly, rather than upon cadaverine. Although no decarboxylase capable of acting upon Δ'-piperideine-6-carboxylic acid is known, further studies may indicate that Δ'-piperideine-6-COOH is decarboxylated to Δ'-piperideine. Cromwell, Roberts, and Webster have demonstrated the presence of a very unstable Δ'-piperideine derivative in pomegranate leaves although this was a propenyl rather than a carboxyl derivative (535). Similar methods (extraction of liquid nitrogen frozen materials) may serve to isolate unstable and transient intermediates in quinolizidine alkaloid biosynthesis.

The possible role of diamine oxidase in the biogenesis of quinolizidine alkaloids remains very much of an open question. Diamine oxidase is known to be present in the plants studied, but a mechanism for generating cadaverine from lysine appears to be absent. It is possible that diamine oxidase may directly oxidize lysine to an intermediate that is decarboxylated to a symmetrical molecule of Δ'-piperideine. Molecules of this reactive heterocyclic compound might then be converted to the quinolizidine alkaloids. Chemical syntheses of quinolizidine alkaloids from Δ'-piperideine have not been demonstrated. A large amount of work remains to be done in elucidating the role of diamine oxidase in quinolizidine biogenesis.

Diamine oxidase has been proposed to play a part in the biogenesis of other, non-quinolizidine alkaloids. The pyrrolizidine and indolizidine alkaloids are very similar to the quinolizidine alkaloids (34, 116).



pyrrolizidine

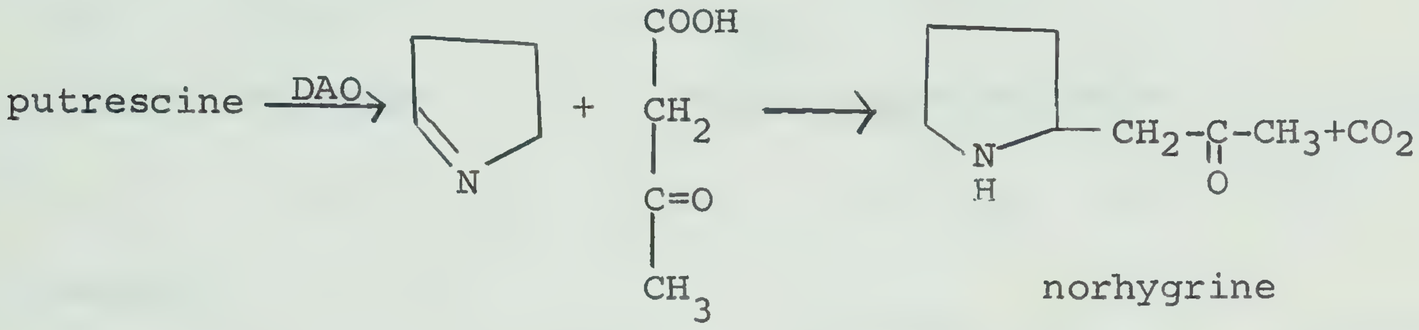


indolizidine

Studies indicate that the pyrrolizidine alkaloids are derived from ornithine via a symmetrical intermediate in a fashion thought to be entirely analagous to that through which lysine is converted to quinolizidine alkaloids (32, 34, 490). It has been postulated that quinolizidine-containing ormosia alkaloids are formed biologically from four lysine units in a similar fashion to the biogenesis of the regular quinolizidine alkaloids (536). Alkaloids related to cryptopleurine (which has a quinolizidine nucleus) are thought to have Δ'-piperideine involved in their biosynthesis (490). If diamine oxidase is important to the biosynthesis of quinolizidine alkaloids, it is also probably involved in the formation of these alkaloids.

Clarke and Mann demonstrated that when putrescine and cadaverine are oxidized by DAO in the presence of β-oxo acids, the β-oxo acids are decarboxylated (330). They found that the output of CO_2 was the same whether the oxo acid is

added during or after the enzyme reaction and concluded that the decarboxylation was a result of the β-oxo acid reacting with the product of diamine oxidation - as they demonstrated in model systems (330). Putrescine and acetoacetate gave (±)-*nor*hygrine and (±)-*iso*pelletierine.



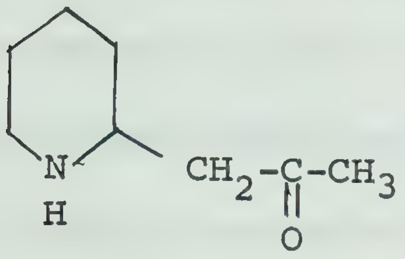
Lysine and ornithine were slowly acted upon by pea DAO to form piperideine and pyrrolidine carboxylic acids that also reacted with β-oxo acids. Various alkaloids could form by such condensations. Clarke and Mann pointed out that amino aldehydes need not necessarily arise through the action of DAO as amino aldehydes might also be formed from lysine and ornithine through the action of such enzymes of amino acid metabolism as ω-hydroxy-α-amino acid dehydrogenases (330). Robinson proposed that coniine family alkaloids such as *iso*pelletierine and methyl *iso*pelletierine could be chemically derived from the diacetals of δ-amino- and δ-methyl-aminovaleraldehydes and acetoacetic acid by mild hydrolysis in physiological pH ranges at room temperature (537). This chemical synthesis has been accomplished. Similarly racemic hygrine and cuscohygrine have been synthesized from γ-methyl

[illegible]

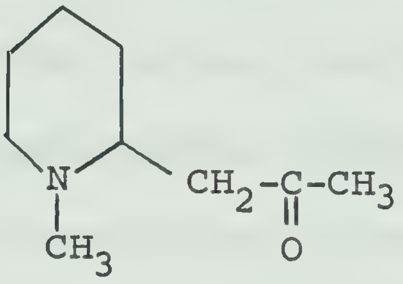
[illegible]

[illegible]

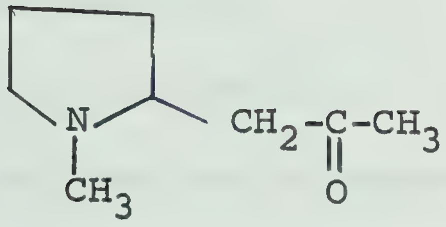
aminobutyraldehyde and acetoacetic acid and from acetone dicarboxylic acid and two molecules of γ-methylaminobutyraldehyde respectively (538).



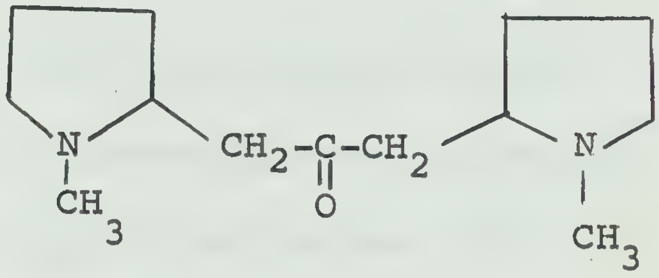
*iso*pelletierine



methyl *iso*pelletierine



hygrine



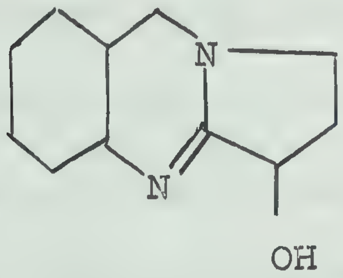
cuscohygrine

These model reactions indicating the possible importance of DAO-derived intermediates in the biogenesis of these alkaloids have recently been studied in detail (539, 540).

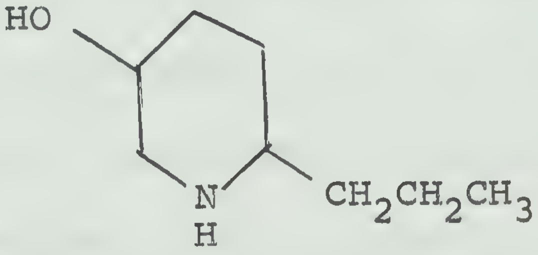
From mixtures of hog kidney diamine oxidase, N-methyl putrescine, and acetoacetate (or acetone dicarboxylic acid) hygrine (or cuscohygrine) were formed respectively (541). N-methyl cadaverine gave *iso*pelletierine and methyl *iso*pelletierine under the same conditions. Hasse and Schmid were able to produce alkaloids through the action of three enzymes that produced amino aldehydes (429). They used pea

DAO, an amine-α-keto acid transaminase from different plant sources, and an amine deacylase from pea and tobacco plants. These workers were able to obtain syntheses of the above discussed alkaloids using these enzymes, acetoacetate (or acetone dicarboxylic acid), and various diamine substrates. There is evidence that Δ'-piperideine is involved in the formation of γ-coniceine in hemlock plants, but there is some doubt that the precursor is derived through diamine oxidase action (355).

Both hog kidney and pea seedling DAOs are able to oxidize the 2-hydroxy derivatives of putrescine and cadaverine to produce hydroxy derivatives of Δ'-pyrroline and Δ'-piperideine (397, 398, 410). Hydroxy-Δ'-pyrroline rapidly converts to pyrroline (398). It has been proposed that these reactions produce precursors of such hydroxylated alkaloids as vasicin and *pseudo*conhydrine (410).

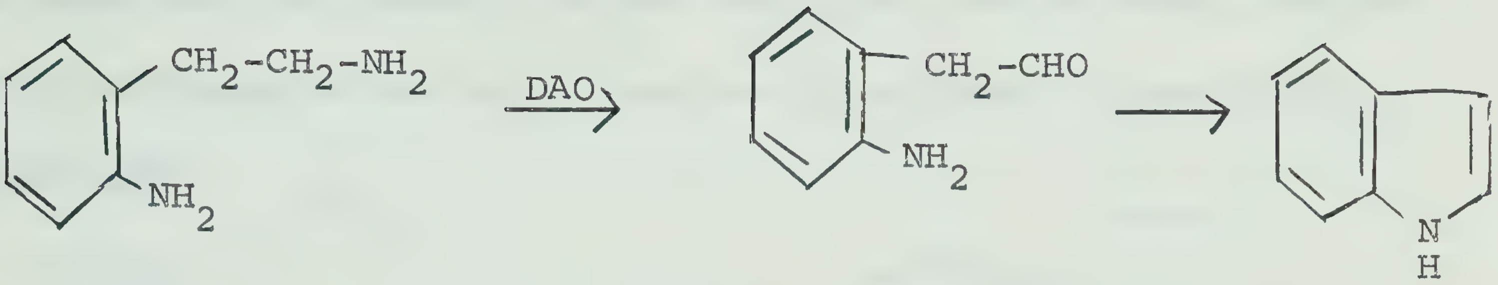


vasicin



*pseudo*conhydrine

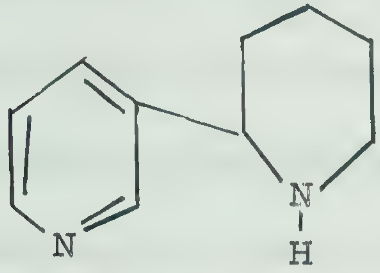
Mann and Smithies have shown that the DAO oxidation of 2-(2-aminophenyl)ethylamine produces indole (332). This might be important in the formation of some indole alkaloids.



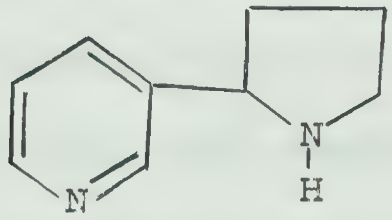
Tracer work indicates that the indole nucleus of many alkaloids is derived from tryptophan (8), but this possibility has not been disproved.

The Nicotiana alkaloid anabasine may be produced from cadaverine after DAO oxidation (531). The Δ'-piperideine produced from cadaverine dimerizes to tetrahydroanabasine which is oxidized by plant extracts to anabasine (421, 422, 531). The synthesis of anabasine from cadaverine -(1,5)-^{14}C by pea extracts has been demonstrated (542). This produces equal, symmetrical distribution of the label in both rings. Leete found that cadaverine fed to Nicotiana plants labeled the piperidine ring, but did not label the pyridine ring (543). Lysine has also been shown not to be the precursor of the pyridine ring of nicotine (34). Furthermore, activity was found only in C2 of the piperidine ring which indicates that free Δ'-piperideine was not involved. It is possible that lysine is incorporated via Δ'-piperideine-2-carboxylic acid (not known to be a product of DAO oxidation of lysine) or that any symmetrical intermediates remain enzyme-bound (34, 543). It is felt to be possible that anabasine may form

in some species through the action of DAO and in other species only the piperidine ring is evolved from lysine (possibly by DAO) (34).



anabasine



nicotine

The pyridine rings of nicotine and anabasine are apparently synthesized from non-amino acid sources (8, 34). The pyrrolidine ring of nicotine is derived from ornithine or glutamic acid (8). DAO has not been suggested in these reactions but evidence does indicate that Δ'-pyrrolidine is involved (8) so DAO is a possibility.

Ornithine (and putrescine) are converted to the tropine moiety of hyoscyamine but the incorporation is asymmetric so a free symmetrical intermediate such as Δ'-pyrrolidine is not involved unless it remains enzyme bound (8, 34, 544, 545). It is thought that a Δ'-pyrrolidine-carboxylic acid intermediate might be involved (8). Such an asymmetric intermediate might result from DAO oxidation of ornithine.

There are a large number of possible roles for diamine oxidase to play in the biogenesis of many alkaloids. Amino-aldehydes, aldehydes, and unstable heterocyclic compounds are all possible intermediates in the formation of certain alkaloids

and they all may be products of diamine oxidase catalyzed reactions, although other enzymes may be responsible for their formation as well. Despite many theories and much speculation there is no evidence for or against the involvement of diamine oxidase in the biogenesis of many alkaloids. The apparent likelihood of DAO participation may vary in different cases but experimental evidence is lacking. Diamine oxidase reactions in most cases are not stereoselective. If DAO is involved stereospecific steps must be found later in the biogenetic pathway.

(4). Interconversions Among the Quinolizidine Alkaloids

Early in the study of the quinolizidine alkaloids it was proposed that oxidized alkaloids such as lupanine and hydroxylupanine were derived by biological oxidations of the sparteine skeleton. Evidence was presented in 1954 that sparteine was converted to lupanine and hydroxylupanine by maturing *Sarothamnus scoparius* seeds (546) and it was later shown that this oxidation was not catalyzed by an ascorbic acid-Fe^{II}-O_2 system (547). Nowacki showed that exogenous sparteine sulphate was converted to lupanine and hydroxylupanine and that this conversion did not take place in alkaloid free legumes (with the exception of *Vicia*, which was able to convert sparteine to lupanine) (548). Lupines were able to convert sparteine to lupanine and lupanine to hydroxylupanine and these

conversions were shown not to be the result of bacterial action in the soil around the roots (235). In 1958 Mironenko suggested that quinolizidine alkaloids have an active metabolic role in lupines (313). Piechowski and Nowacki found extracts of *L. angustifolius* and *Vicia faba* oxidized sparteine to an unknown base at pH 5.6 but not at 7.5 (549). They suggested that a peroxidase was involved. Other workers supplied white lupines with sparteine and found increases in lupanine and hydroxylupanine (248). Increases in lupanine were not followed by similar increases in hydroxylupanine levels. *Lupinus luteus* var. *Popularny* (a sweet yellow lupine) appeared to be able to convert lupinine to sparteine (550). Exogenous sparteine was not converted to lupinine, but was converted slowly to unknown bases (one possibly dehydrosparteine). No conversion of sparteine to lupanine was found (lupanine does not normally occur *L. luteus*) (550). White lupines and beans were able to convert sparteine to lupanine, hydroxylupanine and unknown bases (283). Bitter and sweet types of *L. albus* converted lupanine to hydroxylupanine and other unknown bases (551). The behavior of the two types differed in the rates of conversions and in the proportions of the products. No increase in sparteine was found after lupanine introduction (551). Lupanine-^{14}C fed to white lupines was converted into other alkaloids (but not into sparteine) and was also converted to water soluble and insoluble non-alkaloid

compounds (552). Fodder lupines converted most of the lupanine to non-alkaloid materials while the reverse was true of bitter varieties. Diurnal changes in alkaloid levels were noted by Birecka and Zebrowska (239).

Genetic studies with different *Lupinus* species showed that the oxidations of sparteine to lupanine and hydroxylupanine and of deoxyangustifoline to angustifoline were under genetic control (253). Some species lack the genes for certain of these oxidations and so are unable to accumulate certain alkaloids. Feeding experiments have shown that ^{14}C-lupinine is converted to sparteine (and to lupanine in *L. angustifolius*) in *L. luteus* but sparteine is not converted to lupinine (284, 508). Wiewiorowski and Reifer proposed in 1961 that tetracyclic quinolizidine alkaloids were biosynthesized from higher levels of oxidation to lower, rather than the reverse (553). They considered that angustifoline condensed with formaldehyde to form the C15 quinolizidine alkaloids but presented no experimental evidence for this. An enzyme preparation from sweet white lupines was found to convert lupanine to hydroxylupanine and dehydrolupanine (554). D.M. Graham was unable to confirm these results (personal communication).

Mironenko found interconversions among the alkaloids of *L. luteus* and reported evidence for conversion of alkaloids to non-alkaloid materials (555). Sebyła (250, 251) administered

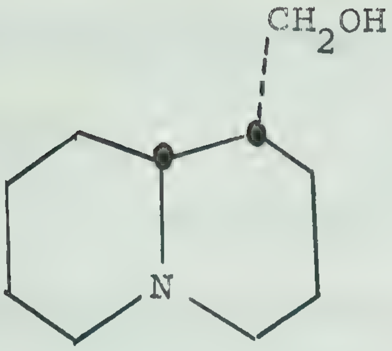
lupanine to white lupines and observed a rise in a fraction consisting of hydroxylupanine esters while there was no increase in the contents of sparteine, angustifoline and of two unknown alkaloids.

Bohlmann, Winterfeldt and Friese (556), in a model reaction demonstrated the formation of all of the natural tetracyclic quinolizidine alkaloids from piperideine and bromolupinine. (The biological equivalents of the latter compound are assumed to be the naturally occurring lupinine esters (556)). Further studies in 1963 favored conversion of sparteine to lupanine, and lupanine to hydroxylupanine and unidentified bases (265, 557). Roots of sweet white lupines slowly converted lupanine to hydroxylupanine, and hydroxylupanine to other, non-alkaloidal materials (558). The suggestion that conversions among tetracyclic alkaloids go from higher to lower oxidation levels received support when conversion of hydroxylupanine to lupanine, in addition to the known lupanine to hydroxylupanine conversion in _L_. _angustifolius_ were reported (559). With _L_. _angustifolius_ plants tracer studies (with tritium labeled alkaloids) on the other hand, gave no indicated that angustifoline or hydroxylupanine could be converted to sparteine or to lupanine, while sparteine was converted to the more oxidized alkaloids (560).

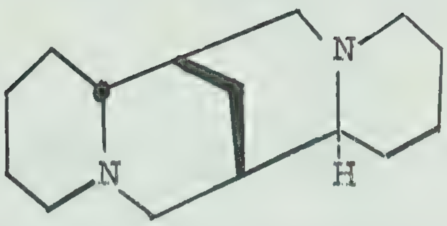
In Genista aetnensis sparteine may be oxidized to retamine, and N-methyl cytisine may be demethylated to cytisine that is then converted to anagyrine (561). Although (-)-sparteine is the major sparteine isomer in this species, traces of (+)-sparteine were found and thought to have been the result of conversions of retamine and anagyrine to sparteine. Plants of Cytisus scoparius are able to form hydroxylupanine directly from sparteine without the intervention of lupanine (562). Conversion of alkaloids to non-alkaloid materials also appears possible. Nalborczyk has further studied the alkaloid transforming principles in extracts of bitter and sweet L. albus and has done some work in purifying the enzyme fractions involved (563). Extracts from Phaseolus vulgaris, Vicia faba, Spinacia oleracea and fodder L. luteus, as well as those from L. albus transformed sparteine, lupanine, hydroxylupanine and angustifoline into their respective dehydro forms. L. albus preparations had no effect upon alkaloids not characteristic of the species (such as lupinine and 17-oxosparteine). Enzyme extracts from bitter and sweet varieties were similar in such properties as the pH, temperature and substrate concentration optima, but differed in the actual rates of conversion. Bitter plants were found to contain a dialyzable inhibitor of the alkaloid-transforming ability; boiled bitter lupine sap inactivated extracts of fodder lupines, but the reverse was not

true (563). Nowacki and Byerrum failed to find tracer evidence for conversion of angustifoline and formate to C15 quinolizidines (155) and therefore favor conversions from lower to higher oxidation levels.

Further studies of quinolizidine alkaloid transformations were carried out using high specific activity tritiated alkaloids (easily exchanged ^3H was removed before use) and sweet and bitter varieties of _L_. _albus_ (564). Sparteine was transformed into dehydrosparteine, lupanine and hydroxylupanine, while lupanine was converted to dehydrolupanine, and hydroxylupanine. When dehydro-alkaloids were introduced, they were converted to hydroxylupanine, lupanine and sparteine. Evidence was also presented for the conversion of alkaloids (particularly dehydroforms) into non-alkaloid materials (564). In 1965 Okuda, Kataoka and Tsuda summarized some of the stereochemical information relevant to the biosynthesis of quinolizidine alkaloids in different genuses (140). They observed that certain stereoisomers are almost always found together. Either (-)-lupinine or (+)-epilupinine is always found to accompany alkaloids of the (-)-sparteine series, as part of the molecule of (-)-sparteine corresponds to the absolute configuration of (-)-lupinine.

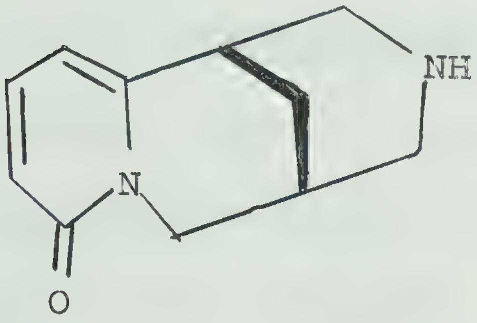


(-)-lupinine

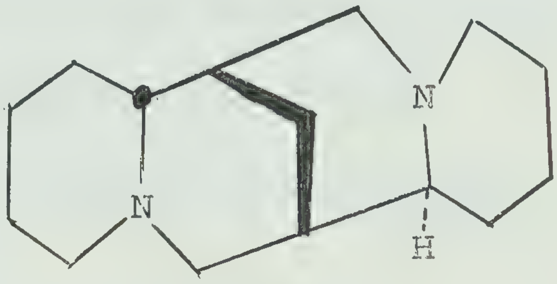


(-)-sparteine

Members of the (-)-sparteine series are often found to accompany (-)-cytisine that has the same configuration of the methylene bridge (140).



(-)-cytisine



(-)-sparteine

Such stereochemical studies will probably afford a great deal of information about the biogenesis of the tetracyclic quinolizidine alkaloids. It may be that valid comparisons of tracer experiments *etc*. can be made only among members of certain genuses known to form alkaloids of the same stereochemical family.

Further genetic studies show that the most probable sequence of interconversions is sparteine → lupanine → hydroxylupanine → hydroxylupanine esters (244, 260). Lack of a gene for the enzyme necessary to one conversion prevents accumulation of alkaloids beyond this point in the species.

The missing gene may be supplied by some hybridizations between species.

Sparteine-^{14}C was converted to lupanine in *L. angustifolius* without any major changes in the carbon skeleton (156). Hydroxylupanine-^{14}C was not appreciably transformed to lupanine and rhombifoline-^{14}C (a non-lupine quinolizidine alkaloid) was not altered or transformed in *L. angustifolius*. In *L. luteus* lupinine-^{14}C is extensively converted to sparteine, but in *L. angustifolius* (which apparently contains no lupinine) exogenous lupinine is not as actively incorporated into tetracyclic alkaloids (156). Some conversion of sparteine to lupinine was observed.

The theory that quinolizidine biosynthesis procedes from higher to lower oxidation states has been enlarged and clarified to include a possible role for the hydroxylupanine ester alkaloids (243, 565). On the bases of chemical similarities and the changes of alkaloid levels during growth and development of *L. angustifolius* it has been proposed that angustifoline and *N*-hydroxymethyl angustifoline are the precursors of lupanine, hydroxylupanine (and its esters) and of α-*iso*lupanine according to the scheme shown in Figure II. The theory involves reaction of formaldehyde and angustifoline to give N-carboxymethyl angustifoline. (This compound, in addition to N-methyl angustifoline and di-N-methyl (di)

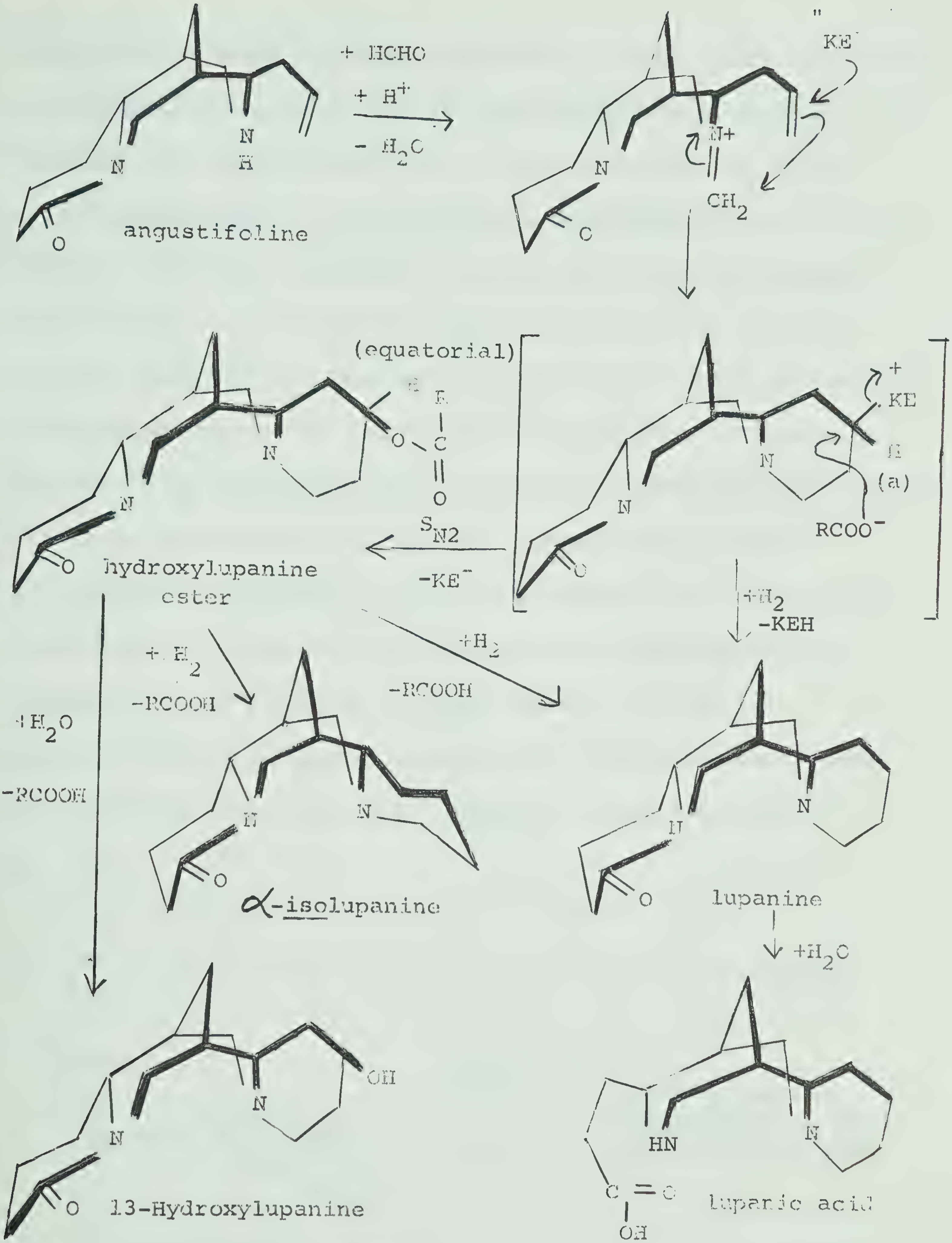
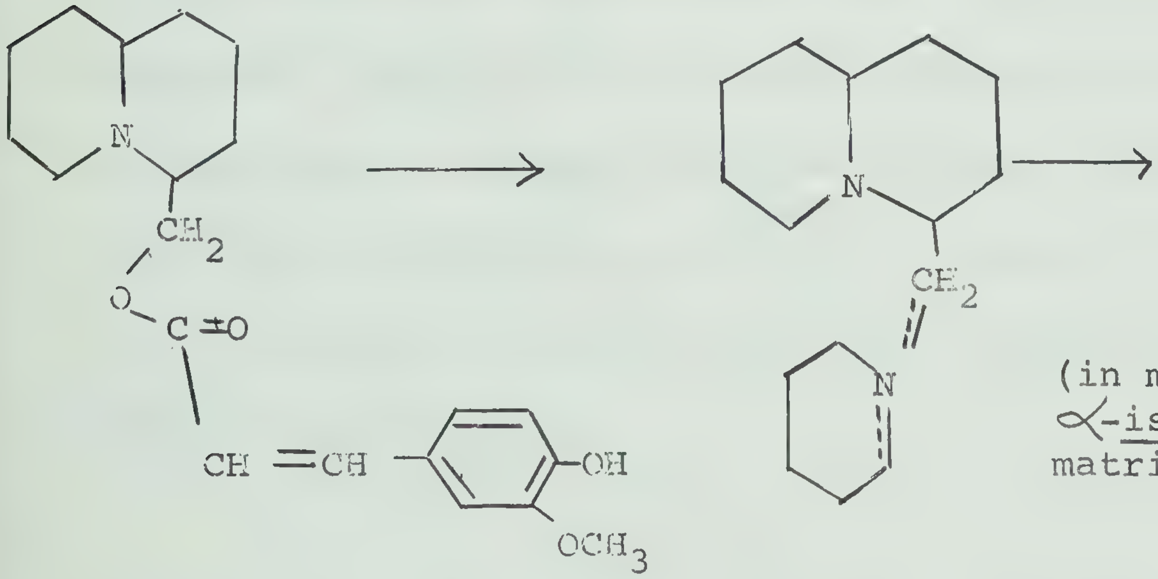


FIGURE II

Hypothetical Pathway from Angustifoline to Lupanine

angustifoline ether have been detected in model reaction systems). N-Carboxymethylangustifoline is then thought to produce an immonium ion that cyclizes to an unstable derivative of 13-*epi*hydroxylupanine. This undergoes a nucleophilic attack by an acid or anion (S_N2 mechanism) to produce 13-hydroxylupanine esters that are converted to other alkaloids (243). They consider that the accepted scheme, sparteine ⟶ lupanine ⟶ hydroxylupanine is not active as they could find no sparteine present in *L. angustifolius*. Other workers have found sparteine traces in this species (240, 523). Esters of lupinine in *L. luteus* are suggested to play an important part in alkaloid formation in this species and the alkaloid dehydroalbine is thought to play a role in *L. albus* similar to that of angustifoline in *L. angustifolius* (565). The proposed scheme for sparteine formation in *L. luteus* is shown below (565):

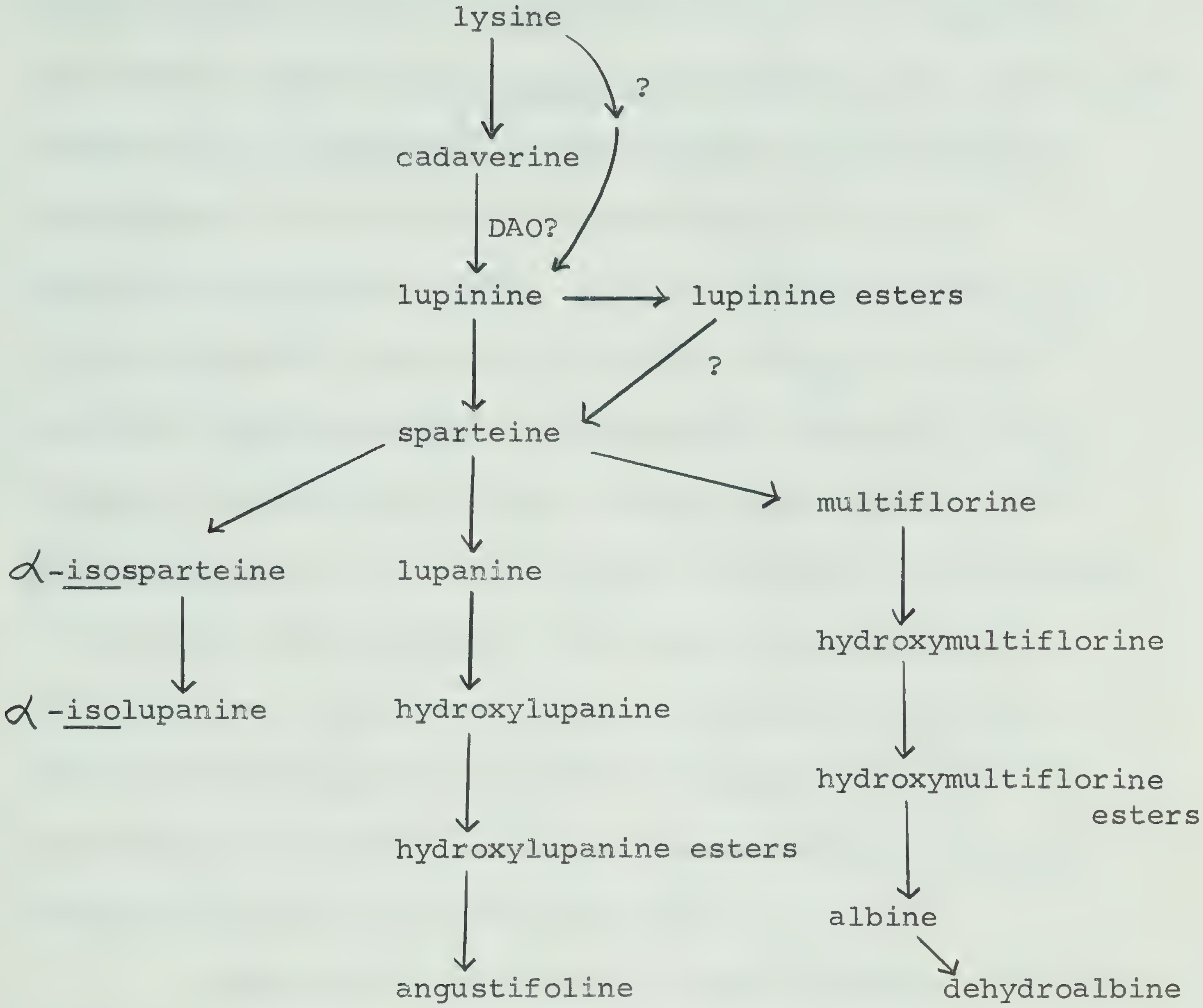


sparteine

(in model systems,
α-*iso*sparteine and
matridine form also

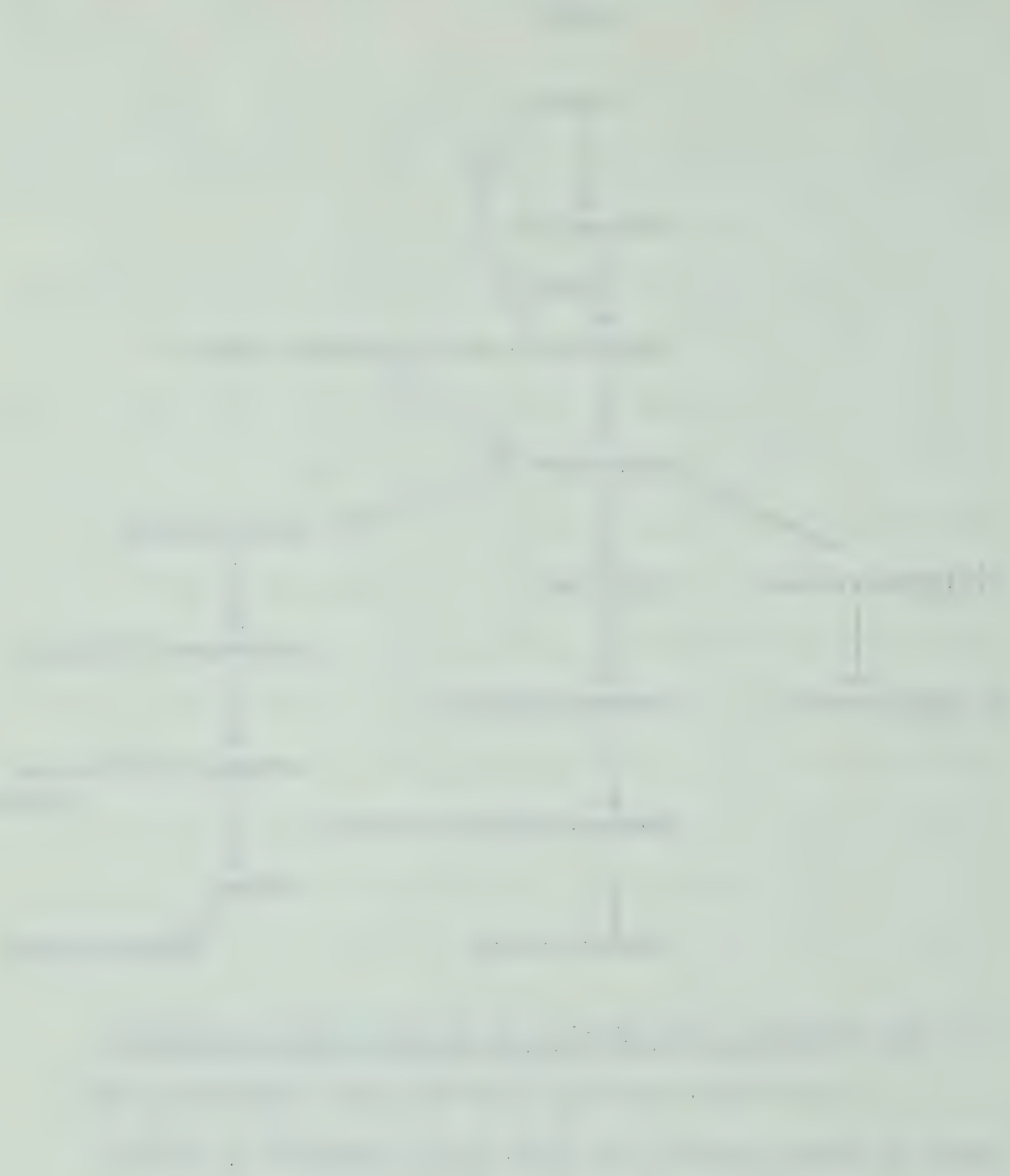
ω-feruloyloxylupinane

An unstable alkaloid found in L. luteus may be a derivative of the intermediate piperidyllupinane (565). The authors of these schemes make no speculations as to the biogenetic origin of angustifoline, dehydroalbine, and the lupinine esters, but an origin from lysine is implicit in the discussion. Biogenetic pathways for the quinolizidine alkaloids are considered by these workers to differ widely among species (243). Genetic evidence does not indicate such a wide divergence although crosses between some species (L. luteus and L. angustifolius for example) cannot be made successfully (156). This theory is not favored by the tracer evidence discussed earlier (particularly by the complete failure of ^{14}C-formate to label alkaloids) nor is it favored by the majority of the alkaloid transformation studies that have been carried out. Until more experimental evidence is available it must be considered that sparteine gives rise to the more oxidized alkaloids and that lupinine is converted to sparteine. The following diagram summarizes the present state of knowledge of the most important, demonstratable conversions among the quinolizidine alkaloids in lupines. (Figure is after references 156, 523, and 529). Each arrow may represent several separate steps.



(5). The Bacterial Degradation of Quinolizidine Alkaloids

Alkaloids diffusing into the soil surrounding the roots of lupine plants are known to be degraded by certain species of bacteria. In 1962 Mozejko - Toczko isolated Pseudomonas lupanini, a strain that aerobically utilizes lupanine as its sole carbon and nitrogen source (566). Sparteine and hydroxylupanine are oxidized only in the presence of lupanine. The intermediates in this degradation are very complex and are quite different from lupanine (568). Both



[illegible]

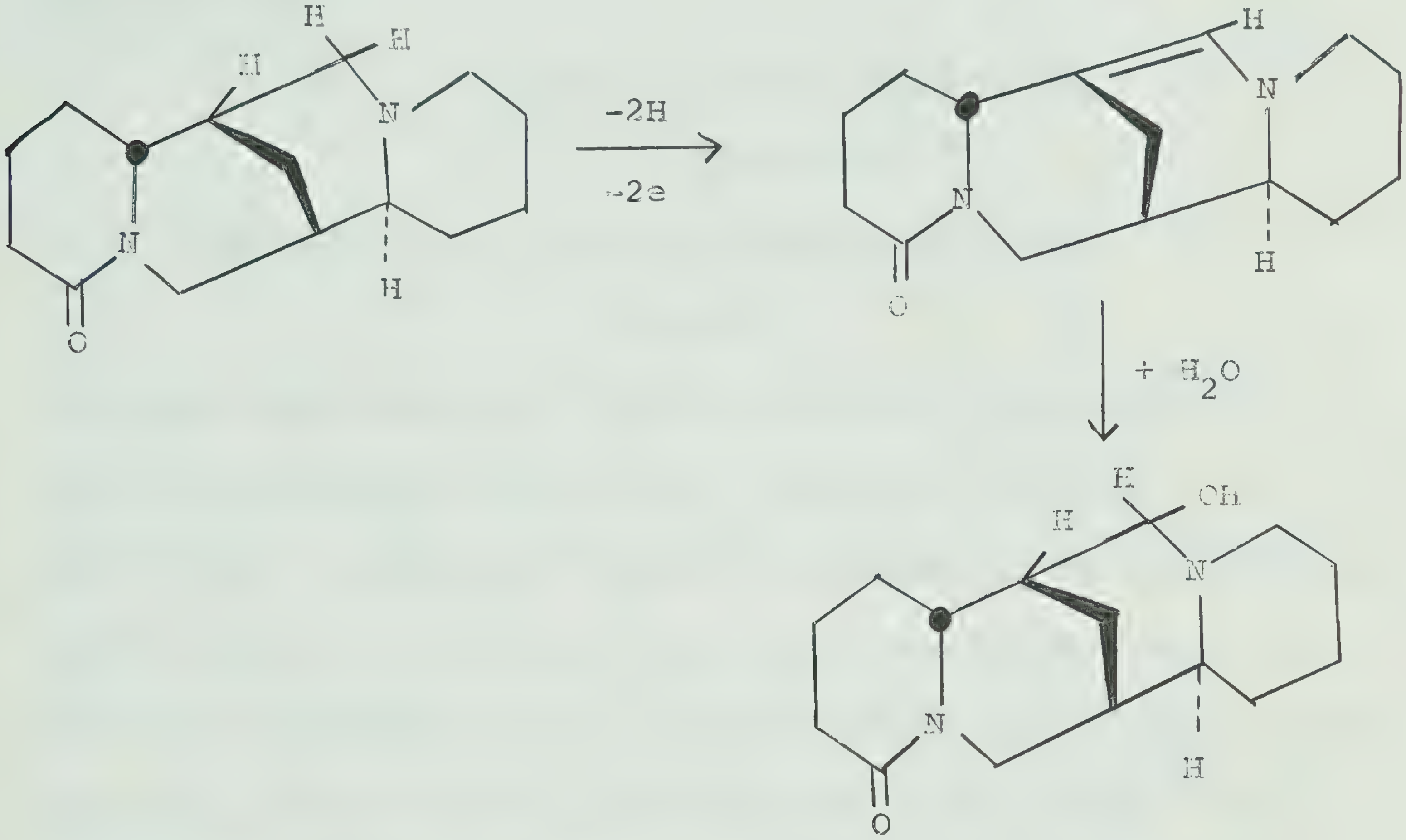
[illegible]

13-hydroxylupanine and 13-*epi*hydroxylupanine are degraded by the enzymes induced in *P. lupanini* by lupanine, but the rate of degradation is slower with hydroxylupanine and an alkaloid intermediate is not further degraded while that from *epi*hydroxylupanine is (567). A final alkaloid product from 13-hydroxylupanine degradation has the ability to induce lupanine oxidizing enzymes; the alkaloid end product from 13-*epi*hydroxylupanine will not induce these enzymes (569). *Epi*hydroxylupanine is therefore not considered an intermediate in lupanine oxidation (569). The major intermediate was identified as 17-hydroxylupanine (not known to be present in other biological materials) (570). Lupanine-N^{16}-oxide is envisaged as a possible oxidation intermediate, or a direct hydroxylation at C-17 could occur (570).

Sparteine ((-) but not (+)) may be used as the sole carbon and nitrogen source by certain strains of *Pseudomonas*, *Corynebacteriaceae*, and *Arthrobacter* isolated from soil surrounding lupine roots (571, 572). Lupinine is decomposed by *Corneybacteriaceae* and *Arthrobacter* strains - the latter also oxidizes lupanine, hydroxylupanine and piperideine (571, 572).

In 1966, lupanine hydroxylyase was isolated and partially purified from lupanine-induced *Pseudomonas lupanini*

(573). The conversion of lupanine to 17-hydroxylupanine required the addition of an electron acceptor and it was proposed that the reaction involved dehydrogenation followed by hydration (573).



G. DIAMINES IN PLANTS

(1). Introduction to Diamines and Polyamines

Several aliphatic polyamino compounds are found in living things. Most important among these are spermidine and spermine (574).

$$NH_2CH_2CH_2CH_2CH_2NH\ CH_2CH_2CH_2NH_2$$

spermidine

$$NH_2CH_2CH_2CH_2NH\ CH_2CH_2CH_2CH_2NH\ CH_2CH_2CH_2NH_2$$

spermine

Of lesser importance are 1,4-diaminobutane (putrescine) and 1,5-diaminopentane (cadaverine). These two diamines (particularly cadaverine) are the chief concern of the following review material. Reference 574 reviews the biochemistry of spermine, spermidine and to a lesser extent, putrescine. Amines in plants are reviewed by Guggenheim (575) who devotes three paragraphs to the occurrence of cadaverine and putrescine in plants.

(2). Chemical Properties of the Diamines

Diamines are basic water and ethanol soluble substances often possessing unpleasant odours. They are commonly used in the form of hydrochloride salts. Both putrescine and cadaverine are bidentate reagents able to chelate such divalent ions as CuII, HgII and CdII (576, 577). The stoichiometry of the complexes is dependent upon pH and

concentration. The ionization constants of the two amino groups are affected by the interactions between the two groups. As the chain length increases, the ionizations become more independent. Cadaverine, with a chain of five methylene groups separating the amino groups, has ionization constants so close together that they are very difficult to resolve by titration (578). The only pK_a values in the literature for cadaverine are $pK_a^1 = 10.25$, $pK_a^2 = 9.13$ at 25° (578). Two sets of values are available for putrescine 10.80, 10.19 for pK_a^1 and 9.35, 8.78 for pK_a^2 (578, 579). Many of the other chemical properties of cadaverine and putrescine resemble those of the amino groups of lysine and ornithine from which these diamines may be derived by decarboxylation.

(3). Isolation, Separation, and Determination of Diamines

Ion exchange resins have frequently been used in the isolation of diamines from tissue extracts and good methods for this are available (580, 581, 582, 583). Steam distillation of strongly basic (*e.g.* 50% w/v NaOH) plant extracts has also been used to isolate diamines from non-basic materials (584).

Separation of cadaverine and putrescine is very difficult. These diamines can be separated by gradient-elution ion exchange techniques (580, 582). Paper chromatography (using specially prepared paper) can separate C2 to C8 diamines (585), and thin layer chromatography of the 2,4-dinitrophenyl

derivatives of some amines has been attempted (586). Most frequently used are electrophoretic methods. Useful paper electrophoresis techniques are described in references 587, 588, and 589. Gas-liquid-chromatography has been used to separate and identify (590) and to determine (591) cadaverine and putrescine. Column packings must be carefully treated with strong bases to reduce polar interactions. Gas chromatography should provide a convenient and sensitive method for the separation and determination of diamines in future studies.

Diamines are generally spectrophotometrically determined as various derivatives. Dinitrophenyl derivatives of amines are readily prepared with Sanger's reagent (dinitrofluorobenzene) (592, 593, 594) or with 2,4-dinitrobenzenesulphonic acid (586). Other derivatives have also been employed (589, 595). Ninhydrin produces a violet colour with cadaverine and putrescine and this can be used as a quantitative method (584, 588). A very sensitive method for the estimation of lysine has been developed that is based upon decarboxylation of the lysine and subsequent formation of N,N'-dinitrophenylcadaverine (596). Spermine and spermidine have been determined by the above methods, but a convenient enzymatic procedure is available (597).

In 1963 Gray and Hartley introduced the use of 1-dimethylaminonaphthalene-5-sulphonyl chloride (DANSYL-Cl) as

a protein end-group reagent (598). Dimethylaminonapthalene sulphonyl amino acids are fluorescent and can be determined in extremely small quantities. The method is 100x more sensitive than the determination of dinitrophenyl derivatives (598). Dansyl derivatives are used for the quantitative determination of free amino acids and electrophoretic and thin layer chromatographic methods are available to separate the various amino acid derivatives (599, 600, 601). Some amine and polyamine dansyl derivatives have been prepared and separated by thin layer chromatography (602). Levels as low as 10^{-4} to 10^{-5} μmoles can be detected. Although dansyl derivatives of cadaverine and putrescine have not been employed, it appears likely that this type of derivative will allow convenient and sensitive determinations and separations of these diamines.

(4). Occurrence of Putrescine and Cadaverine in Biological Materials

(a). Diamines in micro-organisms

Reports of cadaverine in microörganisms are rare. Cadaverine has been found in *E. coli* ribosomes (574, 603, 604). As lysine decarboxylase is present in many micro-organisms (such as *Bacterium cadaveris* and *E. coli*) it appears likely that cadaverine would be present (606, 607).

Putrescine is much more commonly found and is present in fungi and various micro-organisms, (574, 575). Diaminobutane is found in *E. coli* ribosomes (603) and is essential to the growth of *Aspergillus nidulans* (608), *Hemophilus parainfluenzae*, and *Saccharomyces cerevisiae* among other organisms (584). Spermidine and spermine are formed in large amounts by some micro-organisms (574).

(b) Diamines in animal tissues

Cadaverine has not been found to be detectable in many animal tissues (574). There is some evidence for the presence of cadaverine in liver. Although putrescine is more common in animal tissues than is cadaverine, neither diamine is nearly as widely spread as are spermidine and spermine (574). Putrescine is detectable in pancreas, lung, liver and semen.

(c). Diamines in higher plants

Cadaverine (in addition to putrescine, spermidine and spermine) has been found in the seed germs of barley, wheat and other cereals (609). Pea plants exposed to high NaCl levels form cadaverine (610). The volatiles emitted by the flowers of some arum lily species include cadaverine and putrescine (611). Decarboxylation of lysine to cadaverine appears not to occur in *L. angustifolius* (523), but this observation needs careful confirmation. Once again, putrescine has been more frequently found in plant tissues. It is present in citrus

fruit (587), in coconut milk (612), in *Atropa* and *Datura* plants (356), and in potassium deficient barley (613).

(5). Biosynthesis and Metabolism of Cadaverine and Putrescine

The only known mechanism for the biosynthesis of cadaverine is the decarboxylation of lysine. Little decarboxylase activity is detectable in arum flowers so, although free amino acid levels are high, the origin of the cadaverine (and other amines) given off by the plant is a mystery (611). There is no evidence that cadaverine is degraded by the action of any other enzyme save that of diamine oxidase, when cadaverine is administered to mice (437). In 1959, a Ca^{++} activated soluble enzyme system was found in liver that had the unusual ability to incorporate amines (including cadaverine) into proteins with a concommitant release of ammonia (614). The reaction is anaerobic (hence amine oxidases are not involved), is independent of any external energy source, and requires a protein substrate. Incorporated cadaverine can be recovered from thoroughly washed precipitated protein only after hydrolysis. The reaction is thought to be a transamidation between the added diamine and asparagine or glutamine amide groups in the protein (614). Action of such a system *in vivo* has not been reported.

Two pathways for putrescine biogenesis exist in E. coli (605). Ornithine may be directly decarboxylated to putrescine, or arginine may be decarboxylated to agmatine, that then loses the amidine group as urea (through

action of agmatine ureohydrolase) to form putrescine. Putrescine and methionine are the precursors of spermidine and spermine (574). In bacteria putrescine may be degraded by the action of amine oxidases and this oxidation is inhibited by cadaverine (581).

(6). Possible Biological Functions of Cadaverine and Putrescine

(a). Functions related to interactions with nucleic acids

Probably the property of polyamines that is most important in considering their biological functions is the charge they bear in physiological pH ranges. Polyamines have been found to have a strong affinity for nucleic acids (574). In 1958 it was observed that di- and poly-amines (cadaverine being most effective) stabilized an infective agent derived from T2 bacteriophage (615). Cadaverine was thought to interact with the phosphate oxygens of DNA. (At pH 5 the nitrogens of cadaverine are 7.3 Å apart and the phosphate oxygens of DNA are 7.65 Å apart (615)). Cadaverine and polyamines are present in *E. coli* ribosomes although they are readily lost during isolation (603, 604). Cadaverine and putrescine inhibited a ribonuclease present in the preparations (603). Diamines were shown to effect the helix-coil transition of nucleic acids and raised denaturation temperatures by as much as 5° (616). The stabilization effect was shown to be directly proportional to the adenine-thymine content (617) and

cadaverine was the most effective stabilizing agent (618). Many studies have since been performed on the interactions of polyamines with nucleic acids in model systems (619, 620, 621). Attention has been given to the dialdehyde product of the amine oxidase catalyzed spermine oxidation as an inhibitor of some processes involving nucleic acids (622). Diamines are known to affect the coiling of chromosomes during cell division and are so suggested to have a part in the control of mitosis (623).

(b). Diamine accumulation and mineral nutrition in plants

In 1952, putrescine was found to accumulate in potassium-deficient barley plants (613). This accumulation was supressed by sodium and rubidium ions. Exogenous putrescine caused necrosis in barley plants (624). Putrescine was also accumulated in potassium deficient wheat and clover, and disappeared if normal levels of potassium were restored. Accumulated putrescine was slowly metabolized to an unknown compound (624). It has been suggested that in potassium deficiency the normal ionic balance of the cell is disturbed and that organic bases such as putrescine serve to balance the excess of organic acids that would otherwise occur (625). Acid fed to barley plants did increase the levels of polyamines. N-carbamylputrescine amidohydrolase, an enzyme that converts

N-carbamylputrescine (from arginine *via* agmatine) to putrescine, is more active in leaves of potassium-deficient barley plants than in normal plants (626).

Feeding of cadaverine (nutrient containing cadaverine 0.25% w/v) to bean plants inhibited root growth and caused necrotic spots (627). Similar effects were observed when plants were grown on 1% NaCl or 2.1% Na_2SO_4 containing media. Under these conditions sugar accumulation was hindered while abnormally high quantities of amino acids, amides and organic acids were found (627). Pea plants grown on high NaCl media accumulated cadaverine that was thought to lead to the observed accumulations of lupinine, anabasine, trigonelline and other pyridine derivatives (610). Normally peas do not contain measurable levels of lupinine (629). Putrescine is accumulated as well in peas and beans on high NaCl media (628). Diamines in low doses stimulated the growth of broad beans, but at higher concentrations were toxic. Low level of diamine treatment of plants caused a sharp rise in the oxygen absorption and ammonia production of plant homogenates (628). This may indicate the action of an amine oxidase. At toxic diamine levels these features are not observed.

(c). Other possible functions of di- and poly-amines

Aliphatic amines (spermine, spermidine, putrescine, cadaverine) can stimulate cell division in tissue cultures from

Jerusalem artichoke in a manner resembling the results of treatment with naphthalene acetic acid (612). Diamines and polyamines stabilize whole bacterial cells, protoplasts, ribosomes, bacterial cell walls, bacteriophages, and mitochondria (inhibit swelling), as well as prevent inactivation of certain enzymes (574). The mechanisms of these actions are not understood.

(7). Conclusions

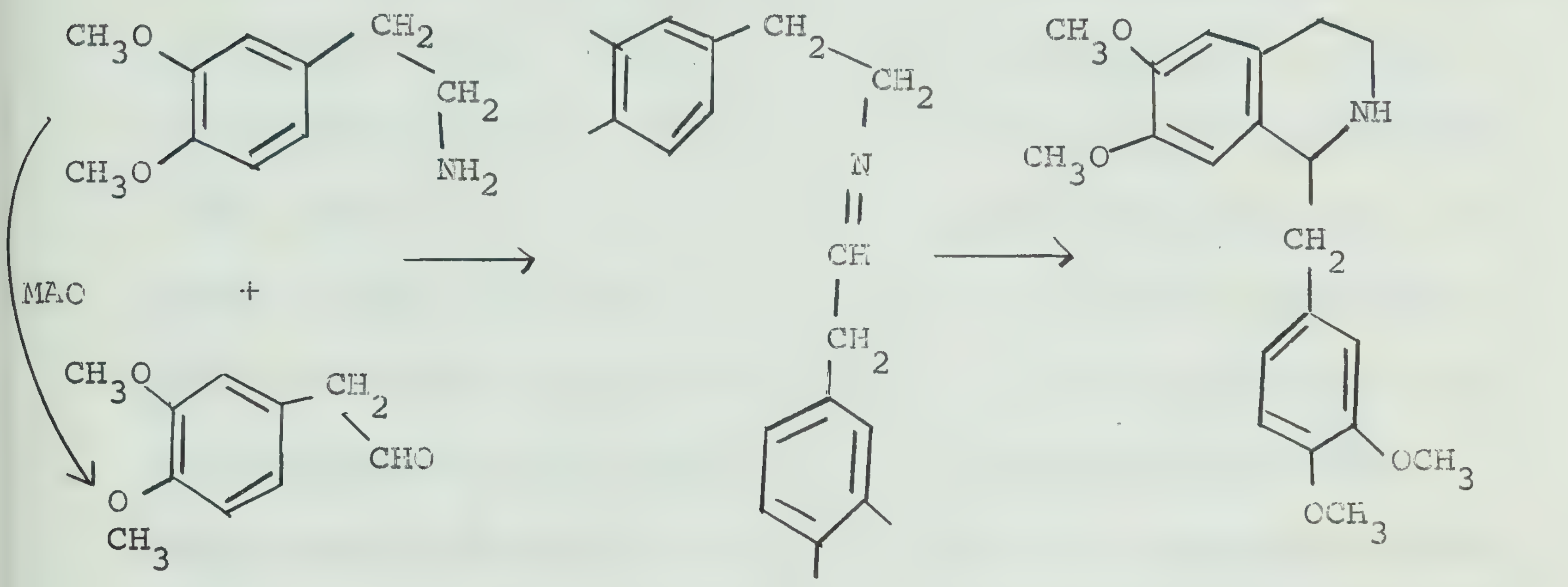
It is apparent that there is really very little known about the biogenesis, metabolism and functions of the aliphatic polyamines in general and particularly of the diamines cadaverine and putrescine. The evidence summarized above indicates that these compounds may have very important roles in growth and development.

The behavior of diamines during potassium deficiency is of particular interest with respect to alkaloid synthesis in lupines. Potassium is known to stimulate lupine growth without increasing alkaloid levels and to counteract the alkaloid increasing effect of phosphorus (see section D6, pp. 90-91 above). Further studies of the interactions of mineral ion levels, diamines and alkaloid formation will possibly lead to a better understanding of alkaloid biogenesis and function. The many alkaloids derived from polyamines (see section F above, and reference 574) may be somehow associated with the interactions of these amines with membranes and with nucleic acids.

H. OTHER ENZYMES POSSIBLY INVOLVED IN THE FORMATION OF QUINOLIZIDINE ALKALOIDS FROM LYSINE

(1). Monoamine Oxidase

Blaschko proposed in 1952 that monoamine oxidase could produce aldehydes that could then undergo coupling with amines to produce certain alkaloids (530).

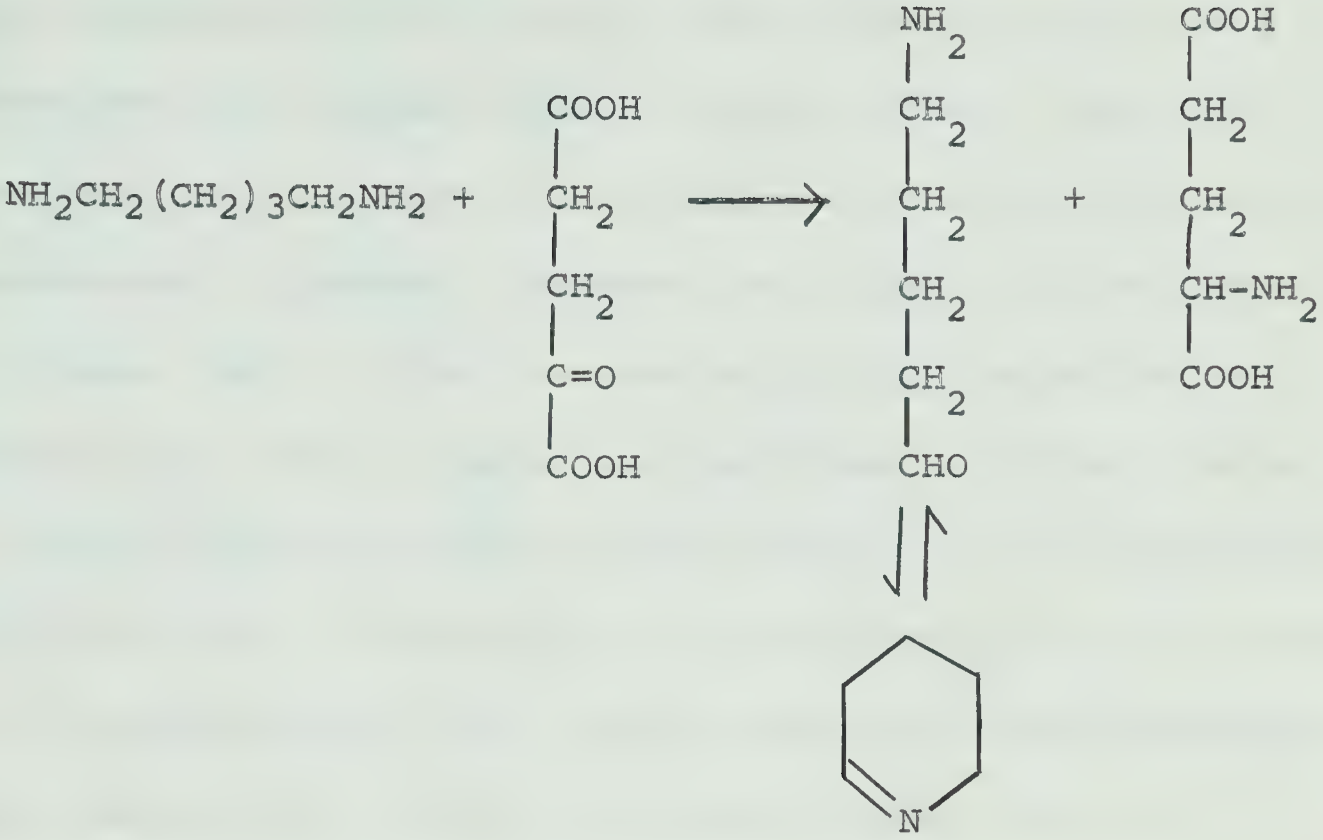


tetrahydropapaverine

As monoamine oxidases can act upon diamines (333, 630) to produce the same products as formed from diamine oxidase action, the possible involvement of plant monoamine oxidase in the biosynthesis of quinolizidine and pyrrolizidine alkaloids must be considered. Werle and Roewer have shown MAO to be present in some alkaloid forming species, but the enzyme is not uniformly present in the plant kingdom (386). Diamine oxidase is present in lupines, but there has been no report of the presence of monoamine oxidase in these plants.

(2). Formation of Quinolizidine Alkaloids through Enzymatic Transamination

Amino acid transaminases are widespread among the higher plants (631, 632). Lupine cotyledons are able to catalyze transamination between α-ketoglutaric acid and alanine, arginine, cysteic acid, α-aminobutyric acid, leucine, cysteine, isoleucine, ornithine, lysine, methionine, γ-aminobutyric acid, glycine, valine and histidine. The amino acids are listed in decreasing (632) order of activity, that with lysine being just over one tenth of that with alanine. Lysine is known to be converted to pipecolic acid in bean plants (633) but the initial product of the L-lysine-α-ketoglutaric acid transaminase from microorganisms is α-aminoadipic-δ-semialdehyde, which immediately cyclizes to Δ'-piperideine-6-carboxylic acid (634). In *L. luteus*, L-lysine is oxidized by a diamine oxidase (directly, not through cadaverine (374) and is not transaminated with α-ketoglutarate, nor is it oxidized by the peroxidase system (377). Various plant extracts (including extracts of lupines and peas) were demonstrated to catalyze transamination between diamines and α-keto acids (particularly α-ketoglutaric acid and pyruvic acid) (635). The product of cadaverine transamination was Δ'-piperideine. This reaction was not reversible, possibly because the aldehyde was present in too low a concentration (635).



The reaction with putrescine was reversible as was the transamination of monoacetyl-cadaverine (transaminated to N-acetyl-δ-aminovaleraldehyde) (635). Certain pyrrolidine and piperidine alkaloids are formed when putrescine and cadaverine are transaminated (with α-ketoglutaric acid) in the presence of plant extracts and β-keto acids (429). The same products may be formed by the action of amine deacylases (in plant extracts) on acylated aminoaldehydes (products of transaminase or DAO actions on monoacylated diamines), or by the direct action of DAO on diamines, both in the presence of β-keto acids (429).

Cromwell and Roberts have suggested that Δ'-piperideine (a possible precursor of γ-coniceine in hemlock) is formed by decarboxylation of the Δ'-piperideine-6-carboxylic acid formed

through transamination of lysine, rather than through lysine decarboxylase and diamine oxidase activity (355). DAO and lysine decarboxylase appear to be absent in hemlock plants, but a transaminase is present. However, no enzyme has been found to be present that is able to decarboxylate Δ'-piperideine carboxylic acid (355). Lysine may be formed in higher plants both through the diaminopimelic acid pathway and the aminoadipic acid pathway (632). Aminoadipic acid (formed from acetyl-coenzyme A and α-ketoglutarate (636)) may be converted to α-aminoadipic-δ-semialdehyde (355), which is in equilibrium with Δ'-piperideine-6-carboxylic acid (this is also the product of direct DAO action on lysine) 427, 428). Δ'-piperideine-6-COOH may be decarboxylated to Δ'-piperideine. Possibly Δ'-piperideine-6-COOH may be converted directly to alkaloids, but in this case some other intermediate would have to provide the randomization of the 2 and 6 carbon atoms necessary in quinolizidine biosynthesis. Hasse has reviewed the possible enzymes involved in quinolizidine formation and considers transaminase involvement to be a distinct possibility (404). He considers that ϵ-N acetyl-lysine might also be a piperideine precursor. Transamination has also been proposed to be important in the biogenesis of piperideines involved in lobelia alkaloid biosynthesis (637). Figure III summarizes the known and possible ways in which lysine may be converted to quinolizidine alkaloid precursors.

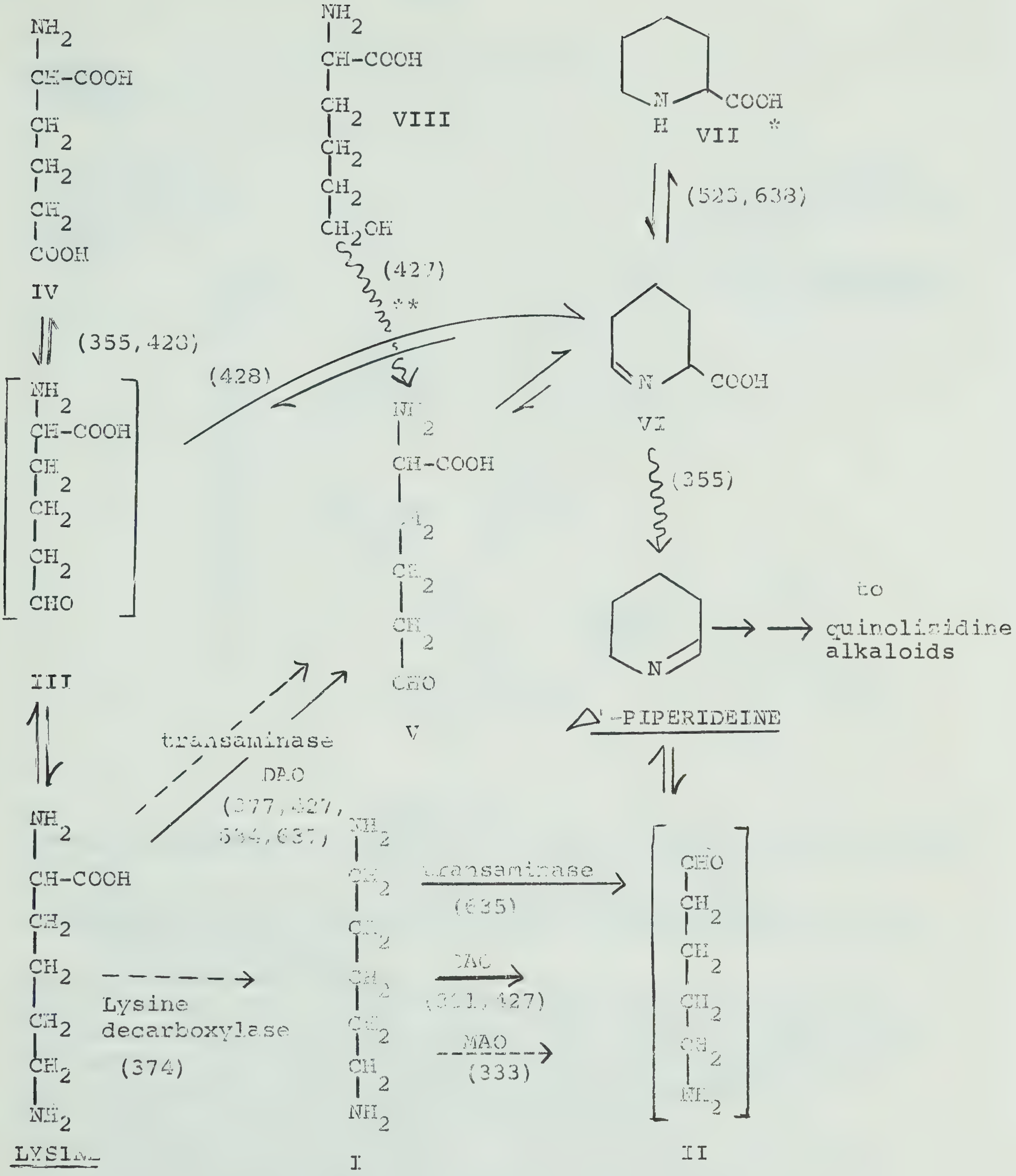


FIGURE III

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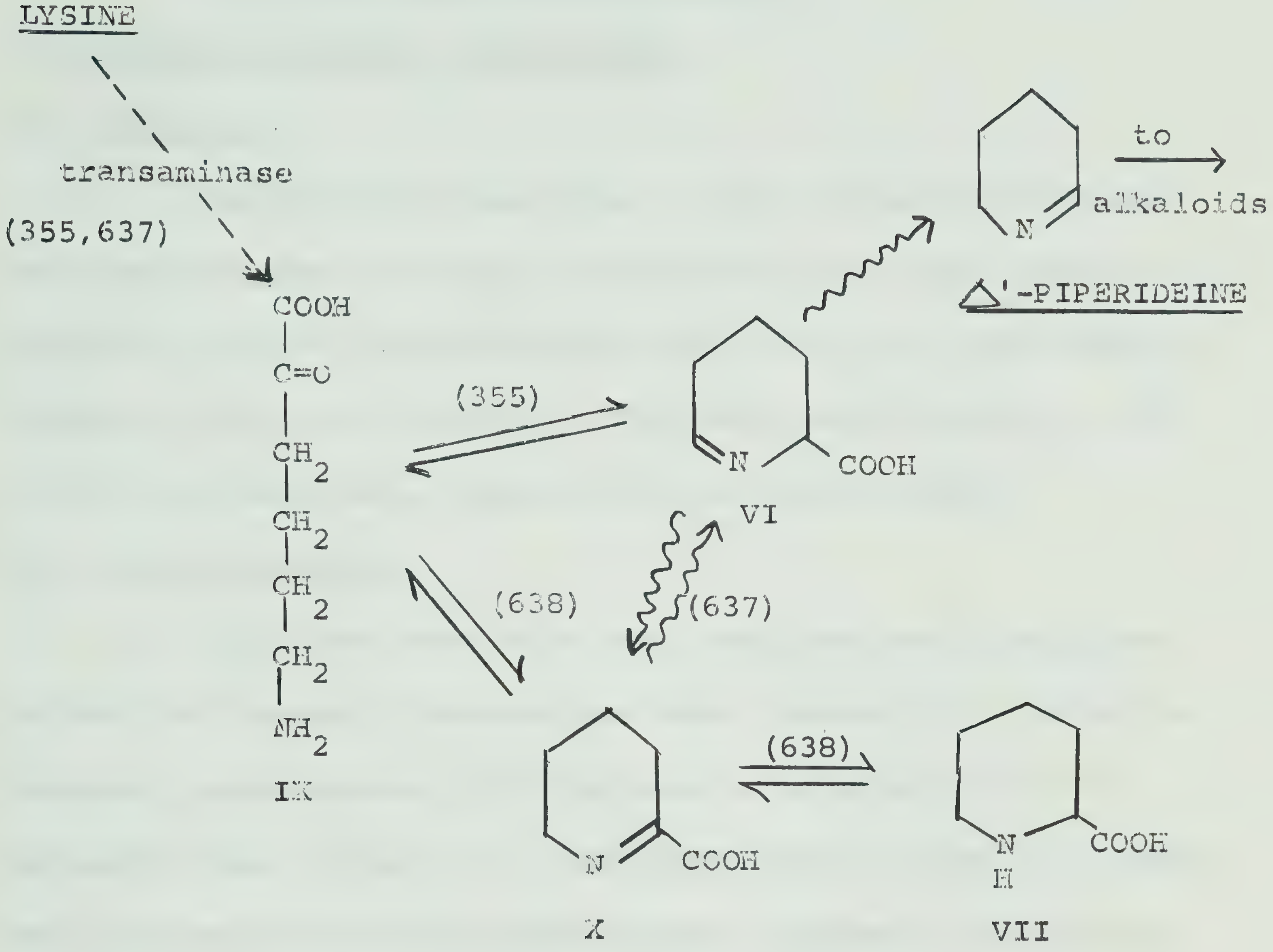

FIGURE III
Possible Conversions of Lysine to Quinolizidine Alkaloid Precursors

——→Reactions known to occur in many higher plants
- - - →Reaction that, although present in higher plants, may not occur in lupines
∿∿→Hypothesized reaction

I: cadaverine, II: δ-aminovaleraldehyde, III: α-aminoadipic-δ-semialdehyde, IV: α-aminoadipic acid, V: α-aminoadipic-δ-semialdehyde, VI: Δ'-piperideine-6-COOH, VII: pipecolic acid, VIII: α-amino-ε-hydroxycaproic acid, IX: α-keto-ε-aminocaproic acid, X: Δ'-piperideine-2-COOH

* Pipecolic acid is formed in legumes (632), but is not a quinolizidine precursor

** ω-hydroxy-α-amino acid dehydrogenase (known in Neurospora)

II. MATERIALS AND METHODS

A. CHEMICALS, ENZYMES, AND SUPPLIES

(1). Alkaloids

All alkaloids employed in this investigation were obtained from Mann Research Laboratories, New York with the exception of free base sparteine which was from Sigma Chemical Company, St. Louis, Missouri. Alkaloids were stored at -15° to -20° and were used without further purification.

(2). Other Chemicals

All other chemicals used were obtained from Fisher Scientific Company, Edmonton and were 'reagent' or 'certified reagent' grade unless otherwise noted. Bio-Rad C-63 ion exchange resin (phosphonic acid type), sodium form, 20-50 mesh, was obtained from BioRad Laboratories, Richmond, California and was purified and converted to H^+ form for use as described by Graham (7, 161). Two different preparations were used: control number C-1, B-1374 (2.95 milliequivalents/ml H^+ resin bed exchange capacity), and control number 3860 (3.1 meq/ml of H^+ resin bed). Aldrich Chemicals, Milwaukee, Wisconsin supplied the o-nitrobenzaldehyde that was used to prepare o-aminobenzaldehyde as described by Smith and Opie (476). The aminobenzaldehyde so prepared melted at 33.5° - 35° and was stored under nitrogen at -15° to -20°. Silica Gel G ('nach Stahl', E. Merck, Darmstadt) was obtained from Canadian Laboratory Supplies, Edmonton.

Cadaverine dihydrochloride (and putrescine diHCl) was "A grade" ("chromatographically homogeneous") and was obtained from Calbiochem., Los Angeles, California. L-lysine monohydrochloride (A grade), D-lysine monohydrochloride (B grade), bovine serum albumin (fraction V) (B grade), isonicotinic acid hydrazide (C grade), pyridoxal phosphate (B grade), and α-ketoglutaric acid (A grade) were also Calbiochem. products. Semicarbazide hydrochloride, p-chloromercuribenzoate, and β-nicotinamide adenine dinucleotide (reduced form) disodium salt (grade III) were obtained from Sigma Chemical Company, St. Louis, Missouri. Chlorpromazine hydrochloride ('Largactil') was the product of May and Baker Ltd., England and was the kind gift of Dr. H.B. Collier. Crystalline human serum albumin was supplied by Pentex Ltd., Kankakee, Illinois. Aminoguanidine bicarbonate (practical grade) was produced by Eastman Organic Chemicals, Rochester, New York. Dimethylaminonaphthalene sulphonyl chloride ('DANSYL-Cl') and ninhydrin were obtained from Pierce Chemical Company, Rockford, Illinois. All of the above chemicals were stored at -15° to -20°.

The following B grade enzyme preparations were obtained from Calbiochem., Los Angeles, California: bovine liver catalase (activity 3,000 Beers and Sizer units/mg.), porcine kidney diamine oxidase (activity 25 µl. O_2/mg./protein/ hour (putrescine) at 37°, pH 7.4), and L-lysine decarboxylase-

Bacterium cadaveris acetone powder (activity 0.22 I.U./mg. at 37°). These enzymes were stored at -15° to -20°. L-Glutamic dehydrogenase was obtained from Sigma Chemical Company, St. Louis, Missouri. The enzyme was a 'type II' preparation, from liver, three times recrystallized and supplied in 50% glycerol-sodium phosphate buffer pH 7.3. The preparation was "substantially free" of ammonium ions and was stored at -5° (above freezing point of solution). The preparation had 10 mg. protein/ml. and 3.1 I.U. per mg. protein.

(3). Solvents

Organic solvents used in these experiments were 'certified reagent' grade and were obtained from Fisher Scientific Company, Edmonton. For gas chromatography and other special applications (where noted) 'certified grade' solvents were carefully redistilled using all glass distilling apparatus equipped with a 40 cm. Vigreux column and protected from atmospheric moisture with a dessicant trap. Anhydrous methanol and ethanol were prepared by distillation from the respective magnesium alkoxides (639). Toluene for gas chromatography work was redistilled from anhydrous calcium sulphate.

The water used for preparing solutions was either demineralized double-distilled water or distilled water that had been passed through demineralizing and organic removal resins before redistillation from an all-glass still. Water

prepared in either fashion never exceeded 2 parts per million ionizable impurities and was frequently well below 1 part per million. Nutrient solution for plant growth was prepared from single-distilled water.

(4). Materials for Scintillation Counting

For DAO assay, and for counting thin layer chromatography spots the standard mixture of the fluors 2,5-diphenyloxazole (PPO) and *p*-bis 2-(5-phenyloxazaloyl) -benzene (POPOP) in toluene was used (640). This was prepared by bringing 42 ml. 'Liquifluor' (obtainable from Nuclear-Chicago Corp., Chicago) to 1 litre with certified reagent grade toluene to give a concentration of 4 g. PPO and 50 mg. POPOP per litre of toluene. PPO and POPOP were also obtained from Nuclear Chicago. Aqueous samples were counted in the naphthalene-dioxane-PPO-POPOP mixture described by Schindler (641). Naphthalene was obtained from Eastman Organic Chemicals, Rochester, New York and was 'high purity' grade. For counting powders removed from thin layer chromatography plates about 0.5 g. Cab-O-Sil was mixed with 15 ml. standard fluor (642). Cab-O-Sil (#M-5) was obtained from the Cabot Corporation, Boston, Massachusettes. Counting was carried out in Nuclear Chicago 'Spectravial II' vials.

(5). Carbon-14 Labeled Compounds

Standardized ^{14}C-toluene was obtained from New England Nuclear, Boston, Massachusetts. The preparation used was lot

#282-155 (catalogue number NES-006) and had a certified activity of 5.26 X 10^5 dpm./ml. ± 3.9%. Other labeled compounds were obtained in 50 μC. amounts from Calbiochem. and all were 'certified' to be better than 99% pure and sample chromatogram scans were supplied by the maker to attest to this. Cadaverine-(1,5)-^{14}C-dihydrochloride (lot 46622) had a specific activity of 4.05 mCi/mmole; L-lysine-6-^{14}C-monohydrochloride (lot 54052) had a specific activity of 2.58 mCi/mmole; and D-lysine-6-^{14}C-monohydrochloride (lot 55694) had a specific activity of 3.0 mCi/mmole.

(6). Supplies for Gas Liquid Chromatography

Gas Chrom Q (80-100 mesh), Chromosorb P (80-100 mesh), stationary phases SE-30, XE-60, DC-560, OV-17, QF-1, and HIEFF-8BP, were obtained from Applied Science Laboratories, State College, Pennsylvania. Hexamethyldisilazane (HMDS) from Pierce Chemical Company, Rockford, Illinois and dichlorodimethylsilane from Matheson, Coleman and Bell, East Rutherford, New Jersey were used in silanization procedures. 'Snoop', a useful leak detecting agent, is obtainable from Nuclear Products Company, Cleveland, Ohio and microliter syringes were the products of the Hamilton Company, Whittier, California.

(7). Some Observations on the Criteria of Purity of Commercially Prepared Chemicals

The purity of the chemicals used in biochemical studies

is often of paramount importance to the successful outcome and correct interpretation of experiments. This is particularly true of tracer studies where a low level contaminant may be used by an organism or preparation to form the compound being studied. Low levels of label in the compound would probably be interpreted as being the result of low level incorporation of the precursor fed rather than having resulted from an active uptake and incorporation of traces of impurity. The most frequently employed test of purity for biological chemistry is paper chromatography. The words 'chromatographically homogeneous' are found on many labels and in many catalogues.

Such a criterion of purity is meaningless unless the solvent system and detection method used are specified. In studies of diamine oxidase it is important to have cadaverine free of other diamines, such as putrescine, yet there are very few paper chromatographic methods that will separate these two very similar compounds. Rarely are details of preparation given. Some preparations might be such as to make the occurrence of certain impurities very unlikely, other preparation methods might make one suspect that chromatographically non-resolvable impurities were very likely.

Some examples illustrating these points have been found in this investigation. D-α-*iso*lupanine was found to give three spots when examined by thin-layer chromatography

(a method more sensitive than paper chromatography). The major spot had the same R_f as sparteine and one of the smaller spots had an R_f value close to that of lupanine. The small conformational difference between the two isomers would be expected to cause only a slight change in chromatographic behavior. It appears safe to conclude that the compound named on the label was actually present as an impurity in some other alkaloid!

Supplied with labeled cadaverine and L and D lysine are copies of geiger-counter scans of chromatograms run with two solvent systems. The solvent systems used to check cadaverine purity were not capable of separating cadaverine and putrescine. In all cases full scale was 30,000 cpm. so impurities of activities much below 1,000 cpm. in the quantity run would not be seen above the baseline, particularly if the spot was diffuse. Chromatography of lysine gives no indication of its optical purity. It is presumed that the methods of purification used were such as to preclude the formation of unresolvable impurities. These ^{14}C-labeled compounds were certified by the maker as better than 99% pure.

Cadaverine-1,5-^{14}C was examined by thin layer chromatography on cellulose and silica gel for impurities. The plate was divided into a 2 X 10 cm. column and each cm. was scraped off and counted as a Cab-O-Sil suspension with a

liquid scintillation counter. No ninhydrin-positive or radioactive impurities were found, however putrescine would not be well separated from cadaverine in the system used.

An enzymic test for the optical purity of D-lysine-6-^{14}C was devised. Lysine decarboxylase is known to be completely specific for the L-isomer (606), therefore lysine decarboxylase would convert to cadaverine any L-lysine present as an impurity in the D-lysine. Pea diamine oxidase in turn oxidizes cadaverine to toluene-soluble materials, which may be extracted and counted by liquid scintillation counting by use of the toluene fluors mixture to extract the product. Details of this method are given below. Although both L and D lysine are oxidized slowly by DAO, the resultant products are not toluene soluble (see results section). Therefore any radioactive materials extracted from D-lysine incubated with lysine decarboxylase and diamine oxidase represent the L-lysine present as an impurity after the appropriate blanks are subtracted. Lysine decarboxylase (1 ml. of 0.2 mg./ml. solution) was incubated one hour at 31° with 0.05 mg. pea DAO, 0.05 mg. catalase, and with 0.2 ml. of L or D lysine-6-^{14}C of 111 X 10^4 dpm./ml. (L-lysine was 0.1938 mM. of 2.58 mCi/mmole specific activity and D-lysine was 0.1670 mM. of 3.00 mCi/mmole specific activity) all in phosphate buffer pH 7.2. The reaction was stopped and the reaction mixture was extracted and counted as described in detail

below. Samples were run in triplicate. Results from blanks containing diamine oxidase and the other components without lysine decarboxylase were subtracted from the results for the complete reaction mixture. Blanks without diamine oxidase gave insignificant levels of extracted radioactivity. The results are summarized below:

	L-lysine	D-lysine
dpm. added	222×10^3	222×10^3
dpm. extracted	22,075.3	346.4
% of radioactivity recovered	9.94	0.16

Only 10% of the L-lysine was decarboxylated and oxidized. This is not surprising as neither the temperature nor the pH were optimum for lysine decarboxylase activity (606). Furthermore, the diamine oxidase reaction does not go to completion (see below). The 0.16% of L-lysine in the D-lysine (assuming specific activities to be the same) is probably correct as the reaction should proceed to completion at such low substrate concentrations. Further work would have to be done to make this test completely reliable, but it does indicate that there is a low, but measurable amount of L-lysine-^{14}C present in the D-lysine-^{14}C. In a feeding experiment where a plant was fed 666×10^3 dpm. of D-lysine-^{14}C, activities of labeled alkaloids would have to be greater than 1,066 dpm. before it could be concluded that D-lysine was capable of being converted to alkaloids.

B. BIOLOGICAL MATERIALS

(1). Seeds

Seeds of *Lupinus luteus* variety *Romulus* were obtained from Thompson and Morgan, Ipswich, England or from Hurst Gunson Cooper Taber Ltd., Witham, Essex, England. Seeds of the sweet variety, *Lupinus luteus Popularny* were the generous gift of Professor Dr. H. Birecka, (Zaklad Fizjologii Róslin S.C.C.W., W. Warozaivie, Poland). Pea seeds (*Pisum sativum* variety *Lincoln Homesteader*) were supplied by Golden West Seeds, Edmonton. All other legume seeds were obtained from Thompson and Morgan Ltd.

(2). Seed Germination

Seeds of *L. luteus Romulus* were soaked for two hours in concentrated hydrochloric acid (10 N NaOH was equally effective but less convenient) and germinated, after thorough washing, on moist white 'Kleenex' tissues in petri dishes for 24 hr. in the dark at room temperature (22° to 27°). Seeds that had swollen were planted. Acid treatment was not required for sweet lupine seeds or for pea seeds. Bitter lupines, sweet lupines and pea generally gave 30 to 50%, 80 to 85%, and 60 to 70% germination respectively after 24 hours.

(3). Growth of Plants

Plants grown on vermuculite or quartz chips were treated every second day with the complete nutrient described

in reference 643, except that iron was added as an FeII-ethylenediaminetetra-acetate chelate instead of being free in solution. Plants being treated with high sodium chloride concentrations were given the above nutrient with appropriate amounts of NaCl added. Such plants were washed with distilled water every three days to prevent accumulation of dry salt deposits on the surface of the quartz chips or vermiculite.

Plants for DAO assay were grown in a growth chamber with a 12 hour light - 12 hour dark cycle. Plants were 18 inches below banks of cool white fluorescent tubes. The temperature was 25° when the lights were on and 18° when they were off. Humidity was uncontrolled but was near 40%. Seeds were planted in 4" x 6" x 2" plastic flats (12 to 18 per flat) in horticultural vermiculite ('Terralite'), which was kept moist with nutrient. Other legumes for DAO testing were grown in the same chamber in 2" x 2" x 2" plastic pots in a mixture of 3 parts soil, 1 part peat moss, and 1 part sand. The soil mixture was autoclaved before use. These plants were given tap water three times a week.

Plants for special experiments were grown in a small laboratory growth chamber that was illuminated 12 hr./day with a bank of 8 fluorescent lamps (alternating cool - white and Sylvania 'Gro-lux' tubes) set 12 inches from the plants. The temperature and humidity in this chamber were not controlled.

The temperature varied between 23° and 30° and was usually between 25° and 27°. The humidity was near 30%. Plants in this chamber were grown either on vermiculite or coarse (1/8 inch) quartz chips moistened daily with nutrient.

Seedlings for diamine oxidase extraction were grown on vermiculite in plastic flats in a dark growth chamber held at 27° and about 80% relative humidity. Flats were kept moist with tap water. Seeds were planted very densly and before use were freed from the vermiculite by washing in a wide mesh screen.

C. SOLUTION PREPARATION

Most solutions used in this investigation were prepared in either M/15 potassium phosphate buffer, pH 7.2 or in M/15 potassium phosphate buffer, pH 7.2, containing 0.2 mg./ml. bovine serum albumin. The abbreviations 'KPB' and 'BSA-KPB' respectively are used in the following text to refer to these buffers. Where concentration and/or pH were varied, note is made of this. In absence of information to the contrary, concentration of buffer was M/15, pH was 7.2 and serum albumin concentration was 0.2 mg./ml.

All solutions containing protein and/or phosphate buffer were stored frozen at -15° to -20°. For short term use solutions were stored at 0° to 6°. It was found that BSA-KPB solutions were particularly subject to mold and

bacterial growth and care was taken to avoid the use of solutions so contaminated.

D. ISOLATION AND PARTIAL PURIFICATION OF PEA AND LUPINE DIAMINE OXIDASE

(1). Diamine Oxidase Isolation and Purification

The purification method employed was adapted after that of Mann (389). As the procedure at each step was slightly modified from the original, full details are given below. The figures in brackets after each step are the weights, volumes *etc*. found in a typical lupine DAO preparation. Except where noted, all centrifugations were carried out in an International Equipment Company B-20 refrigerated centrifuge.

Etiolated seedlings 7 to 10 days old were washed free of vermiculite, blotted dry, chilled, and coarsely ground with a chilled Phillips 'Gourmet Centre' meat grinder (200 g. of *L. luteus Romulus* seeds gave 698 g of seedlings - of average weight 1.0 to 1.2 g., 9.5 cm long stem, and 4 to 6 cm. root). All further operations were done at 0° to 6° except where noted. The pulp was squeezed through 4 layers of cheesecloth and the residue was homogenized for 2 min. in a Waring Blendor (full speed) with 1/2 ml. per gram of seedlings pH 7.0 cold KPB. The mixture was squeezed through five layers of cheesecloth and pooled with the fluid previously obtained. The pH of the

cold homogenate was brought to pH 7 ± 0.1 with potassium hydroxide (850 ml. of homogenate at pH 6.4 at 5° was obtained before pH adjustment).

To the cold extract was slowly added 0.3 ml./g. seedlings 2:1 ethanol (absolute)-chloroform at -22° with rapid stirring. The mixture was then stirred for 30 min. and centrifuged 20 min. at 5,500 g [872 head]. The supernatant was carefully separated from the chloroform and precipitate (1,025 ml. at pH 7.0). To the supernatant 45 g./100 ml. enzyme grade ammonium sulphate Mann Research Laboratories, New York and the mixture was stirred 9 hr. Centrifugation for 20 min. at 11,000 g. [872 head] gave a floating 'precipitate' that was removed with a spatula and by pouring the supernatant (1,160 ml.) through a single layer of cheesecloth. The precipitate was ground to a smooth paste with a mortar and pestle with 1/2 ml./g. seedlings 0.02M pH 7.0 KPB (300 ml.). The suspension was stirred 2 hours at 23° and 9 to 10 hours at 0° to 6°. (The pH of such a suspension was 6.8 at 0°). The suspension was centrifuged 20 min. at 6,000 g. [872 head] and the pellet was stirred with a small amount of 0.02M pH 7.0 KPB and recentrifuged.

To the combined supernatants (390 ml.) was added 18 g./100 ml. ammonium sulphate and the mixture was stirred 1 to 2 hours before centrifugation at 6,000 g. for 20 min. To the supernatant (435 ml.) was added a further 24 g./100 ml. ammonium

sulphate and the mixture was stirred for 1 to 2 hr. The resulting suspension was centrifuged 20 min. at 22,400 g. [model L-2-50 refrigerated ultracentrifuge, Beckman Instruments, Spinco Division, California, #19 head]. The pellets were dissolved in a small amount (40 to 50 ml.) of 0.02M pH 7.0 KPB and the supernatant (490 ml.) was discarded.

The enzyme solution was dialyzed in 1 1/2 inch diameter cellulose tubing for 8 hr. against running tap water [15°] and then against 2 x 6 litres of 0.005M pH 7.0 KPB [0° to 6°] for a total of 36 hr. The dialysate (143 ml.) was centrifuged 20 min. at 10,000 g. [870 head] and the supernatant was frozen with dry ice-acetone and lyophilized at 5 μ (microns) of Hg pressure with a Virtis 'Unitrap' freeze-dryer [Fisher Scientific, Edmonton].

The powder was dissolved in 40 ml. cold deionized water and the pH (originally 7.02) was brought to 5.00 at 5° with 0.05N acetic acid. The pH 5 solution was allowed to stand about 10 hr. before being centrifuged for 20 min. at 10,000 g. The supernatant (59 ml.) was discarded and the pellet ground in a mortar and pestle with 20 ml. deionized water. The pH of this suspension was brought to 7.00 at 5° with 0.05N potassium hydroxide and was centrifuged 20 min. at 10,000 g. The supernatant was again brought to pH 5.0, allowed to stand, and centrifuged as above. The pellets again were ground, brought to pH 7.0, centrifuged, adjusted to pH 5.0, centrifuged,

ground, brought to pH 7.0 and recentrifuged to give a solution of diamine oxidase (31 ml.) that had been precipitated at its isoelectric point a total of three times. This solution was frozen with dry ice-acetone and lyophilized. The final powder was stored at -15° to -20° above anhydrous calcium sulphate.

Samples were taken at each stage of the above procedure for DAO assay and protein determination.

(2). Protein Determination

Protein concentrations were determined with the Folin-Ciocalteu phenol reagent (644). Samples were diluted with water to give readable protein levels. The procedure employed was that described in reference 644 for proteins readily soluble in NaOH solutions except that it was found necessary to prepare the carbonate copper solution by mixing 100 ml. 2% (w/v) sodium carbonate in 0.1N NaOH, with 1 ml. each of 2% (w/v) sodium tartrate and 1% (w/v) cupric sulphate to avoid precipitation. Samples were read in 1 cm. matched quartz cuvettes against a reagent blank at 500 mμ and 750 mμ with a Beckman DU-2 spectrophotometer. A standard curve was prepared using known concentrations of crystalline human serum albumin in water.

E. ASSAYS FOR DIAMINE OXIDASE ACTIVITY

(1). Diamine Oxidase Assay after Holmstedt and Tham

Holmstedt and Tham developed a method for

spectrophotometric assay of DAO based on the formation of coloured compounds after reaction of o-aminobenzaldehyde with the reaction products of DAO oxidation of cadaverine and putrescine (419, 425). Assay conditions were adapted for assay of plant DAO. The following mixture was incubated 3 hr. at 37°: 0.005M o-aminobenzaldehyde in KPB pH 6.8 (stored under N_2 at 0° to 6°) 2.5 ml., KPB pH 6.8 3.5 ml., 0.1M diamine (putrescine or cadaverine) freshly prepared in pH 6.8 KPB or water (blank) 0.5 ml., DAO solution 1.0 ml. After incubation, to each sample was added 1 ml. of 10% (w/v) aqueous trichloroacetic acid. Centrifugation was found to be unnecessary and samples were read at 430 mμ (putrescine) or 450 mμ (cadaverine) in a Beckman DU-2 spectrophotometer.

(2). Polarographic Measurement of Oxygen Consumption

Oxygen consumption as a result of diamine oxidase activity was followed with a Clark type polarographic electrode-the Model 53 Biological Oxygen Monitor Yellow Springs Instrument Company, Yellow Springs, Ohio. A Beckman 100 mV. potentiometric recorder (10 inch scale) operated at 0.5 inches per minute provided a permanent record. Probes were prepared and the instrument operated as outlined in the instruction manual supplied with the instrument. It was not found possible to prepare probes that met the 87% O_2 saturation criterion as outlined in the manual, but probes coming to 84-85% were found

entirely satisfactory. The sample chamber was held at 24° and all additions were made of solutions at 24°. A total volume of 3 ml. in the chamber was employed and the relative amounts of buffer and diamine oxidase varied. Additions of substrates were made with 100 µl Hamilton liquid syringes with pieces of fine gauge polyethylene tubing attached to the syringe needles. For computational purposes, an oxygen concentration of 225 µM for air-saturated phosphate buffers is used in our laboratory (645).

(3). Diamine Oxidase Assay by Scintillation Counting of the Reaction Products from ^{14}C-Cadaverine

The method developed by Okuyama and Kobayashi (426) was studied thoroughly and adapted for use with plant diamine oxidase. This method was extensively used in this investigation. Only by careful attention to small details can it be made to yield good results. With such attention to detail the technique is reliable, versatile, sensitive and the results are reproducible. The details described below are those developed during the course of studies with diamine oxidase and bear little resemblance to those used by the original developers of the method.

Solutions of cadaverine-(1,5)-^{14}C dihydrochloride in pH 7.2 KPB were prepared for use as substrates. All solutions used (except where noted) contained 1.776×10^5

dpm./ml. Small aliquots of each solution were counted to check this. Cadaverine concentrations were 0.5, 1.0, and 5.0 mM having specific activities of 0.160, 0.082, and 0.017 mC./mmole respectively. These solutions were stored frozen at -22°.

Test tubes equipped with screw plastic caps (with white rubber liners) were used for incubation and extraction. These tubes were 13 X 100 mm., 10 ml. capacity and may be obtained from any laboratory supply house. About 20% of the tubes of any given batch fail during the freezing and centrifugation steps when they are first used. It is therefore a useful procedure to fill new tubes with water and subject them to freezing and centrifugation. After the weak tubes have been removed, breakages fall to an insignificant levels. Caps were checked before use, and those with loose liners were discarded.

The total reaction mixture volume was always 2.0 ml. A typical sample would consist of 1.0 ml. DAO solution (or of buffer for the blanks), 0.1 ml. of 0.5 mg./ml. catalase in BSA-KPB, 0.8 ml. BSA-KPB, and (added at start of run) 0.100 ml. cadaverine-^{14}C solution. When inhibitors or activators were added the amount of BSA-KPB was reduced to 0.3 ml. and 0.5 ml. of inhibitor in KPB or BSA-KPB was added. Samples were preincubated in a water bath set at 30° to 35° for 30 minutes. The water bath available was extremely difficult to adjust,

therefore the incubation temperatures varied somewhat; however runs of a similar nature were done at the same temperature. The temperature during a run varied from that stated no more than ± 1°. Samples were removed from the bath in groups of four at 3 min. intervals and 0.100 ml. substrate solution was added to each tube with a Hamilton liquid syringe. Tubes were immediately capped, mixed by inversion, then the caps were allowed to drain for 1 min. The caps were then removed and the samples replaced in the bath. At all times caps and tubes were kept matched to avoid contamination of one sample with part of another. At the end of 1 hr. incubation time, tubes were again removed in groups of 4 and about 0.2 ml. of a saturated solution of aminoguanidine bicarbonate in one quarter saturated sodium bicarbonate (at 23°) was added to each tube. This was followed by the addition of sufficient sodium bicarbonate (200 to 300 mg.) to saturate the solution and to leave layers at the bottoms of the tubes. Next 5 ml. of toluene-PPO-POPOP was added with a 5 ml. syringe and tubes were capped and were shaken briefly. The above procedure reduced error in the 60 min. incubation of each sample to less than 1 min. When the reaction was stopped in all of the tubes they were either shaken for 1 min. violently by hand (when less than 72 tubes were used) or were shaken 5 min. with an Eberbach Corp., Ann Arbor, Michigan reciprocating shaker

operated at full speed. (When 72 tubes were used they could be conveniently mounted in the shaker). Equivalent results were obtained from both methods of shaking the tubes. The emulsions that formed during shaking were broken by full speed centrifugation of the tubes for 6 to 12 min. in either an International Equipment Company HN centrifuge, a Measuring and Scientific Equipment Company clinical centrifuge, or in a Sorvall angle centrifuge with SPX head operated at 70% of full speed. After centrifugation samples were examined to assure that a layer of sodium bicarbonate was present in each. The tubes were then placed in a deep freeze at -22° for 1 to 1.5 hr. to freeze the lower, aqueous layer. Longer periods in the freezer tended to break some test tubes. The toluene layers were poured off into scintillation counting vials, the samples were melted, 5 ml. more toluene-PPO-POPOP was added and the shaking, centrifugation and freezing steps were repeated. The whole operation was repeated a third time to collect 15 ml. of toluene solution for each sample that was then counted in a scintillation counter. Blanks were frequently run to correct for unoxidized cadaverine extracted. When any other materials, such as various inhibitors, were added, separate blanks were run with these to correct for any changes caused by the added material in the background level of cadaverine extracted.

(4). Scintillation Counting and Quench Correction Procedures Used

In liquid scintillation systems, energy is frequently lost before it reaches the photomultiplier tubes. This phenomenon is known as quenching and the degree of quenching often varies with the particular nature of the sample being counted. Thus, if samples with dissimilar degrees of quenching are to be compared, some method of correcting the cpm. observed to dpm. must be used. The most reliable method for the determination of the counting efficiency is the internal standard method (646). When this procedure is employed the samples are first counted and then a known amount of a standard isotope solution (^{14}C-toluene is frequently used) is added and each sample is recounted. This permits determination of the counting efficiency which may then be used to convert the sample cpm. to dpm. This procedure was used to prepare quenching curves (see below) and to determine the counting efficiency of particularly important samples. Toluene-^{14}C was used and the certified 5.26×10^5 dpm./ml. solution was carefully diluted ten times with redistilled 'certified reagent' toluene to give a solution of 5.26×10^4 dpm./ml. ± 4.2% (i.e. ± 2,210 dpm./ml.). To each sample being checked was added 50 µl. of this standard solution (i.e. 2.63×10^3 dpm. ± 5.2% (± 137 dpm.)) with a 50 µl. fixed needle Hamilton syringe. The use of a Hamilton syringe has been shown to be completely satisfactory for such work (646).

For routine work efficiency correction was done with the use of external standardization. The counters used were equipped to irradiate the sample vial with γ-radiation from barium-133 (in Nuclear-Chicago instruments) or from caesium-137 (in Beckman instruments) for a short time. The energy spectrum of the Compton electrons produced by such irradiation resembles that of C-14 β-particles and should be quenched in a similar fashion to a similar degree. The net external standard counts (as the full activity is known) provides a crude measure of counting efficiency. A much more reliable method of determining the degree of quenching is to take the ratio of the external standard counts in two channels set to monitor different portions of the energy spectrum. Highly quenched samples will record fewer counts in the low energy channel than in the high. Thus, the external standard channels ratio will vary with the degree of quenching. The counting efficiency can be related to the external standard channels ratio by counting a set of differently quenched samples containing a known amount of internal standard. From the 'Quenching Curve' so obtained, the counting efficiency of any channel may be determined from the external standard channels ratio. Quenching resulting from colour in the sample will vary greatly with the particular sample counted. It is therefore best to prepare a quenched series of samples by use of the same sort of quenching agents present in the samples

themselves. This is contrary to the usual procedure of counting a series of acetone-quenched samples supplied by the manufacturer and assuming that the relation between the external standard channels ratio and the efficiency is the same as that for the samples.

Three instruments were used in the present investigation: two were Nuclear Chicago Mark I instruments (one in the Animal Science Department, one in Plant Science) and the third was an LS-200B model from Beckman Instruments (Dept. of Plant Science). Quenching curves were prepared for each instrument used. Operational details for the two types of instruments are given below.

Two channels were used in the Nuclear-Chicago Instruments - one was "optimized" for ^{14}C counting and the other for ^{32}P counting. Both were operated in the integral (L-U) mode with the upper discriminator gate set at its highest voltage and the lower gate set at its lowest voltage. ^{14}C counts were taken from the ^{14}C optimized channel and the ratio of external standard counts in the ^{14}C optimized channel to the ^{32}P optimized channel was taken for quench correction. (It would have been preferable to have different discriminator settings for each channel and to optimize one channel for external standard counting). The sample chamber was held at 8°.

The Beckman LS-200B was equipped with two channels used exclusively for external standard counts and the ratio between these two was printed out. One channel was used for ^{14}C counting with a ^{14}C 'Isoset' to set the discriminator gates. The samples were counted at ambient temperature (near 25°).

Toluene solutions containing large amounts of any quenching agents present in the DAO assay extracts were prepared. A typical preparation follows: 18 g. of 7 day old yellow lupine plants were ground with 50 ml. BSA-KPB. The homogenate was centrifuged to remove the larger particles. Lupine homogenate (50 ml.) was incubated at 31° for several hours with 0.25 mmoles cadaverine, 1 mg. bovine catalase, and 5 mg. pea DAO (all in BSA-KPB). The mixture was saturated with sodium bicarbonate and extracted with 4 X 100 ml. toluene-PPO-POPOP. Emulsions were broken by centrifugation and the yellowish toluene phase was poured through cotton to remove suspended water. Various amounts of this quenched fluor mixture were placed in scintillation vials and the sample volume was brought to 15 ml. with unquenched toluene-PPO-POPOP. These samples were counted to determine individual backgrounds and then internal standard was added and the samples were recounted.

Sample vials and caps were cleaned carefully. Before evaporation of the toluene from emptied vials, they were rinsed

with ethanol (95%) containing 1% (v/v) concentrated HCl. Vials were next rinsed with tap water, then scrubbed in hot soapy water, and again rinsed with tap water. An overnight soaking in hot soapy water followed by tap water and distilled water rinses and oven drying completed the washing procedure.

(5). Glutamic Dehydrogenase Linked Diamine Oxidase Assay

The velocity of the glutamic dehydrogenase catalyzed synthesis of glutamate from ammonia and α-ketoglutaric acid is proportional to the ammonium ion concentration at low ammonia levels (as a result of the high K_m of glutamic dehydrogenase for ammonia) (472). The velocity of this reaction may be measured by following the oxidation of NADH. This method for ammonia determination has been adapted by Lorenz, Kusche and Werle for the assay of diamine oxidase activity (444). The following procedure was used for assay of plant diamine oxidase activity.

Into two matched, 1 cm. quartz cuvettes were added 1.0 ml. of pH 7.2 sodium phosphate buffer (NaKPB) 0.2M, 0.100 ml. of 5.7 mM disodium NADH in NaKPB (freshly prepared) 0.100 ml. 0.171M α-ketoglutaric acid, 0.2 ml. glutamic dehydrogenase solution containing 5 mg. protein/ml. with a specific activity of 3.1 I.U./mg. protein in NaKPB containing 25% (v/v) glycerol, and 1.0 ml. DAO solution in KPB or KPB-BSA. These components were mixed and 0.5 ml. NaKPB was added to one cuvette as a

blank and, at zero time, 0.5 ml. substrate solution (e.g. 1.8mM cadaverine in NaKPB) was added to the other cuvette. After being briefly stirred the samples were read at 3 min. intervals at 366 mμ* in a DU-2 spectrophotometer (Beckman Instruments) equipped with a deuterium lamp. The sample compartment was held at 24.0° by use of spacers through which water from a constant temperature bath was circulated. Readings were taken until the reaction had stopped.

F. EXTRACTION OF DIAMINE OXIDASE FROM PLANTS

(1). Qualitative Extraction in Survey of Legume Genuses for Diamine Oxidase Activity

Plants of various quinolizidine-alkaloid forming and non-forming genuses and species were examined for DAO activity. Plants were harvested three months after planting. When sufficient tissue was available it was pressed as described below and the fluid so exuded was diluted with KPB-BSA and frozen. When only a small amount of tissue was available the tissue was gound in a 15 ml. conical centrifuge tube with a glass rod in 2 ml. KPB-BSA. Samples were centrifuged, stored frozen at -22° and diluted 5X or 10X for use. Treatment following this was that described below.

(2). Quantitative Extraction of Diamine Oxidase from Plant Tissues

Bitter and sweet yellow lupines and pea plants were examined for diamine oxidase activity. The method developed is

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* Wavelength used by originators as a matter of convenience, not of necessity.

elaborate but was intended to keep DAO concentration high, to give complete extraction, and to permit examination of activity resulting from both easily and difficulty extractable diamine oxidase.

Typical plants of each age were harvested at the same time each day. Plants were carefully washed and weighed. Some were frozen for alkaloid analysis while others were used for DAO extraction. For diamine oxidase extraction 2 1/2 to 3 1/2 g. of plants were weighed out and were pressed between two chilled 6 inch diameter, 3/4 inch thick lucite discs that had a 1/4 inch X 1/4 inch groove running 1/2 inch from the perimeter. A Wabash laboratory press (model 12-10-2) was used to press the tissue between these plates. The tissue was pressed briefly, then one disc was rotated 180° with respect to the other and pressure was applied for 3 min. The pressure used was between 20,000 and 23,000 lbs. (load on a 2 inch diameter ram). Fluids pressed out of the tissue were collected in the groove and could be removed with a pipet. Examination with a phase contrast microscope of tissue so pressed revealed that the majority of the cells were disrupted. Only the fibrous materials appeared little affected by the treatment. An aliquot (1.0 ml.) of the expressed fluid was removed and brought to 25.0 ml. with KPB-BSA. A 10 ml. aliquot of this suspension was centrifuged with an International HN centrifuge at full

speed and then stored frozen at -22° as an 'A' sample. The pulp was moistened with a small amount of cold KPB-BSA and was pressed again. The fluid expressed was diluted 1 ml. in 25 ml. and treated as above ('B' sample). This procedure was repeated a third time to obtain a 'C' sample. The pulp was next transferred to a 15 ml. conical centrifuge tube and was ground (using a glass rod) with 10 ml. cold KPB-BSA. The homogenate was centrifuged and the pulp extracted again with 10 ml. KPB-BSA. This extraction was carried out a total of eight times. The 80 ml. of extract was diluted with KPB-BSA to 100 ml. and a 10 ml. aliquot was centrifuged and frozen as above ('D' sample).

Additional plants were examined for DAO activity present in the fluid expressed by the first pressing. Such samples were prepared as the 'A' samples described above and were known as 'squish' samples. All samples were capped with parafilm and stored at -22°.

Before DAO assay, samples were thawed and centrifuged for 20 min. at 80,800 g. with a L2-50 Beckman preparative ultracentrifuge using a #40 rotor (35,000 rpm.). Samples were held at 0° to 6° during these operations. The supernatants were carefully poured off into clean tubes and were frozen until use. Some samples (A and B samples from germinating seeds) had a buoyant 'precipitate' and in such cases the upper

2 ml. of sample was removed by suction before the supernatant was poured off. Later in the course of this work it was found that freezing the bottoms of the centrifuge tubes by immersion in dry ice-acetone effectively immobilized the pellets and the supernatants could be poured off without concern for carryover of pellet materials.

During all stages of the above procedure, care was taken to work rapidly, to keep the samples cold, and to work as quantitatively and reproduceably as was possible.

G. THIN LAYER CHROMATOGRAPHY OF QUINOLIZIDINE ALKALOIDS:

TLC TECHNIQUES

A method, more satisfactory than any described in the literature, was developed for the thin layer chromatographic (TLC) separation of some of the quinolizidine alkaloids.

Layers of silica gel G (250 μ thick) were prepared on 20 cm. square glass plates with the coating apparatus described by Stahl (obtained from Canadian Laboratory Supplies Ltd., Edmonton) (170). For five plates 25 g. silica gel G was shaken with 50 ml. deionized water for 30 sec. in a plastic bottle before application. Plates were air dried for a minimum of 24 hours before use. Cellulose-coated plates were prepared by homogenizing 10 g. of Machery and Nagel MN-300 cellulose powder (available from Canadian Laboratory Supplies Ltd.) with 60 ml. deionized water for 2 min. in a Sorvall Omnimixer operated at

full speed. Scrupulously clean plates were necessary for successful coating (especially with cellulose). Plates were scrubbed with a commercial cleansing powder (such as 'Ajax') and were carefully rinsed with tap water and deionized water.

Alkaloids were spotted either from acidic solutions of the alkaloid salts or from solutions of the free bases in dichloromethane, by use of capillary micropipets.

Plates were developed with a mixture of chloroform, acetone, methanol and 28 to 30% (w/v) aqueous ammonia in the proportions 15:20:3:1. The solvent mixture was prepared immediately before use. The walls of the developing tank were thoroughly wetted with the solvent before development of the plate. Solvent migration was stopped by a line ruled through the layer 10 cm. from the origin. Plates were dried at 70 to 100° for 1 hr. before development. Dragendorff reagent (Munier-Macheboeuf modification) was prepared as described in reference 170. This reagent was stored at room temperature (647) and was discarded when it became dark red instead of orange in colour. Prior to treatment with Dragendorff reagent, plates were frequently sprayed with 1% (w/v) iodine in chloroform to enhance the staining. Plates were heated briefly after this treatment to remove excess iodine.

H. EXTRACTION OF ALKALOIDS FROM LEGUME SEEDS

Seeds from the same batches of seed used for the legume plants grown for DAO activity examination were extracted to remove any alkaloids present. Such extracts were examined by TLC for the presence of alkaloids in an attempt to correlate the presence of alkaloids with the presence of diamine oxidase.

About 10 seeds (or an equivalent weight of larger seeds) of the various legumes were ground to a powder with a mortar and pestle. The powder was transferred to a 15 ml. conical centrifuge tube where it was ground with 3 drops of 14-15% (w/v) aqueous ammonia. The moistened powder was mixed with 2 ml. dichloromethane and allowed to stand 30 min. After centrifugation the organic solvent layer was removed and stored in a small corked test tube at -15 to -20°. Approximately 20 µl. of each extract was spotted on a silica gel plate which was developed and stained as described in the preceding section. Varieties that gave no detectable alkaloid were reëxamined. A larger number of seeds was ground and extracted as above and the extract was reduced by evaporation under a stream of nitrogen to a few tenths of a milliliter. The concentrated extract was spotted in 50 to 70 µl quantities on TLC plates. The extraction method described above was a modification of that reported by Cranmer (27).

III. RESULTS AND DISCUSSION

A. A NOTE ON OTHER EXPERIMENTS

Time did not permit the inclusion in the main body of the thesis certain parts of the experimental work done on this problem. The results, as well as details of methods and materials, for these experiments will be issued under separate cover as appendices to this thesis. The appendix contains a discussion of the use of gas chromatography to separate and determine quinolizidine alkaloids, details of experiments in which lupine and pea plants were fed L- and D-lysine-6-^{14}C and cadaverine-(1,5)-^{14}C, a discussion of the separation of sparteine and lupinine by ion exchange chromatography, some titration data for some quinolizidine alkaloids, details of an attempt to determine diamines by the preparation of their dansyl-derivatives, separation and detection of diamines from lupine tissue, and some general observations and experiments about lupine growth.

B. QUENCHING CURVES

Figure IV shows the relationship between counting efficiency and the external standard ratios obtained with a Mark I Nuclear-Chicago Scintillation counter and a series of quenched standards prepared as described in section II. The spread of the points is such that a given external standard channels ratio permits a corresponding efficiency to be read

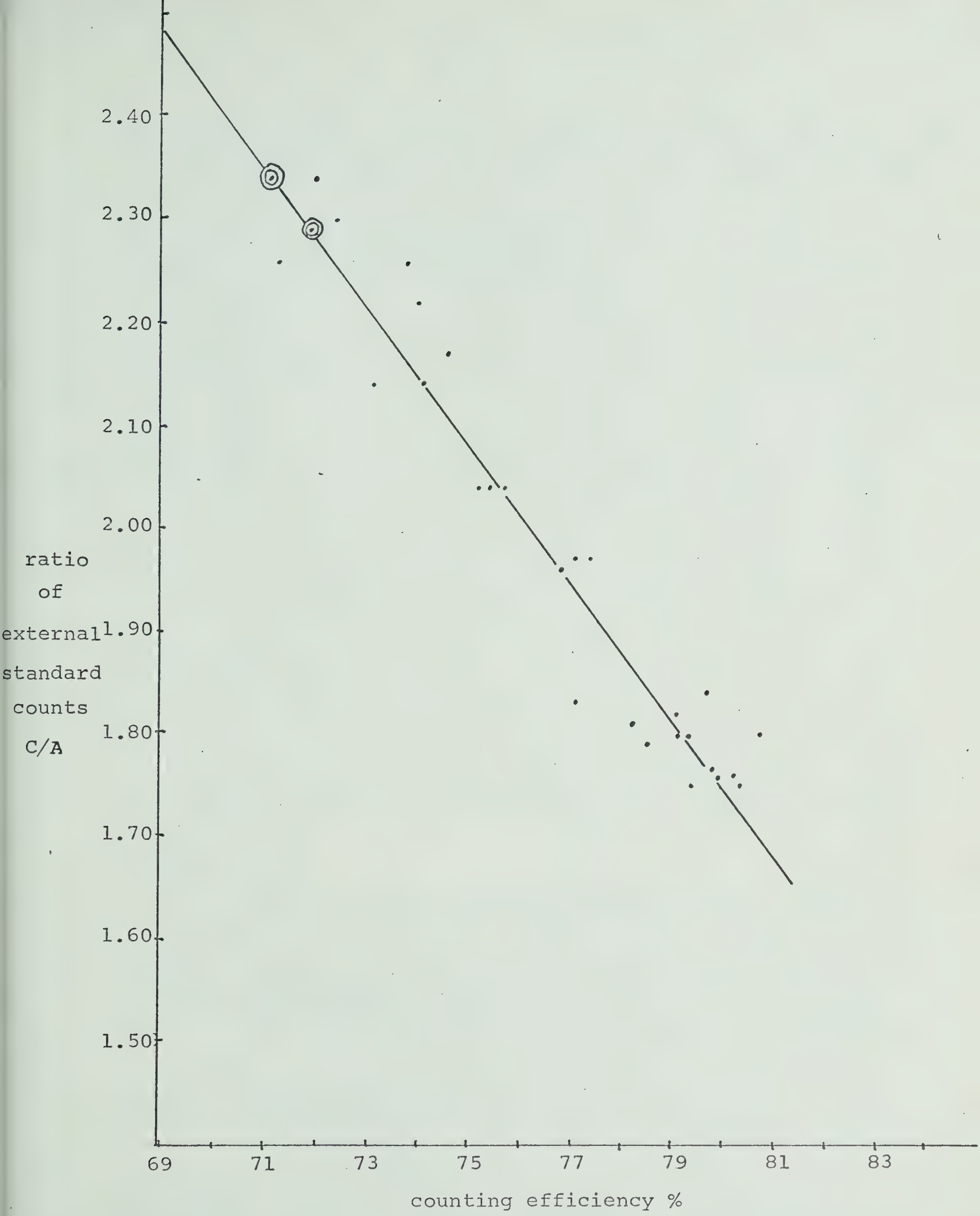


FIG. IV: Quenching curve for diamine oxidase assay method. Samples quenched with quenching mixture described in methods section. Efficiencies determined by addition of ^{14}C toluene standard.
Nuclear-Chicago mark I, L-U, L = 0.0, U = 9.9
attenuators A = G : 6.40, C = D : 9.12, time 20 min.

to ± 2% efficiency. This is quite acceptable performance. When a series of quenched standards prepared by Nuclear-Chicago were examined, a typically curved plot resulted. These standards ranged from 88% (logarithm_{10} of external standard channels ratio = 2.24) to 28% efficiency (logarithm of external standard channels ratio = 6.49). The most highly quenched sample that could occur in a DAO assay sample gave 71% counting efficiency (external standard channels ratio = 2.45) and the least quenched sample (*i.e.* no quenching agent added gave 81% counting efficiency (external standard channels ratio = 1.88). It is apparent that little quenching material is extracted into the toluene during DAO assay. Over such a short range, it is not unusual that the quenching curve appears to be linear. Figure V shows that there is a good linear relationship between the amount of quenching mixture added and the external standard channels ratio.

The Beckman LS-200B liquid scintillation counter has a less satisfactory external standard system. Figure VI shows that the relationship between the external standard channels ratio and the amount of quenching agent is not linear and that there is somewhat more scatter in the points. Table VI gives the external standard channels ratios for a series of quenched standards, with the efficiencies obtained. It is apparent that there is no clear relationship between the counting efficiency

ml. quenching agent (prepared as outlined in section II)	external standard channels ratio	% Efficiency
0.0	0.87	98.4
0.0	0.86	97.8
0.0	0.86	97.1
1.0	0.82	97.6
1.0	0.80	97.2
1.0	0.80	97.2
3.0	0.71	96.2
3.0	0.75	97.2
3.0	0.76	97.3
5.0	0.71	101.1
5.0	0.71	101.7
5.0	0.72	99.0
6.0	0.66	95.7
6.0	0.66	95.0
6.0	0.67	93.9
7.5	0.64	97.4
7.5	0.66	97.2
7.5	0.65	95.9
10.0	0.60	98.3
10.0	0.62	98.1
10.0	0.61	97.6
12.0	0.57	96.8
12.0	0.58	96.0
12.0	0.58	97.1
14.0	0.56	95.9
14.0	0.56	95.5
14.0	0.57	95.4
15.0	0.53	96.8
15.0	0.54	94.3
15.0	0.55	94.6

TABLE VI. Counting efficiencies and external standard channels ratios for a series of quenched standards counted 20 min. on a Beckman LS-200B liquid scintillation counter

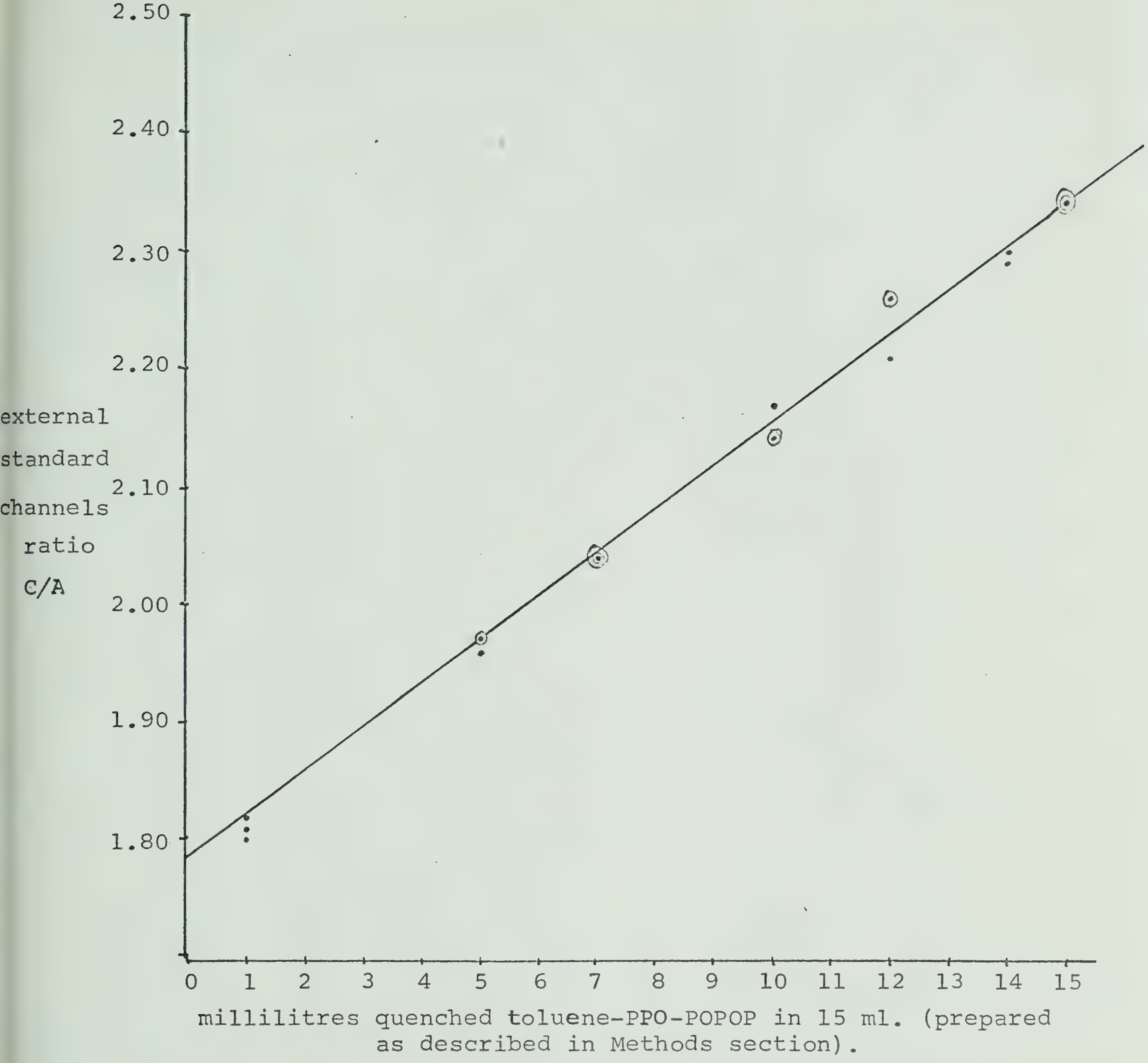


FIG. V: Relationship between external standard channels ratio and amount of quenching agent added.
Nuclear-Chicago mark I Scintillation Counter.
attenuators: C = D : 9.12, A = G : 6.40
mode: L-U, L = 0.0, U = 9.9
time: 20 min.
Circles represent coincident points.

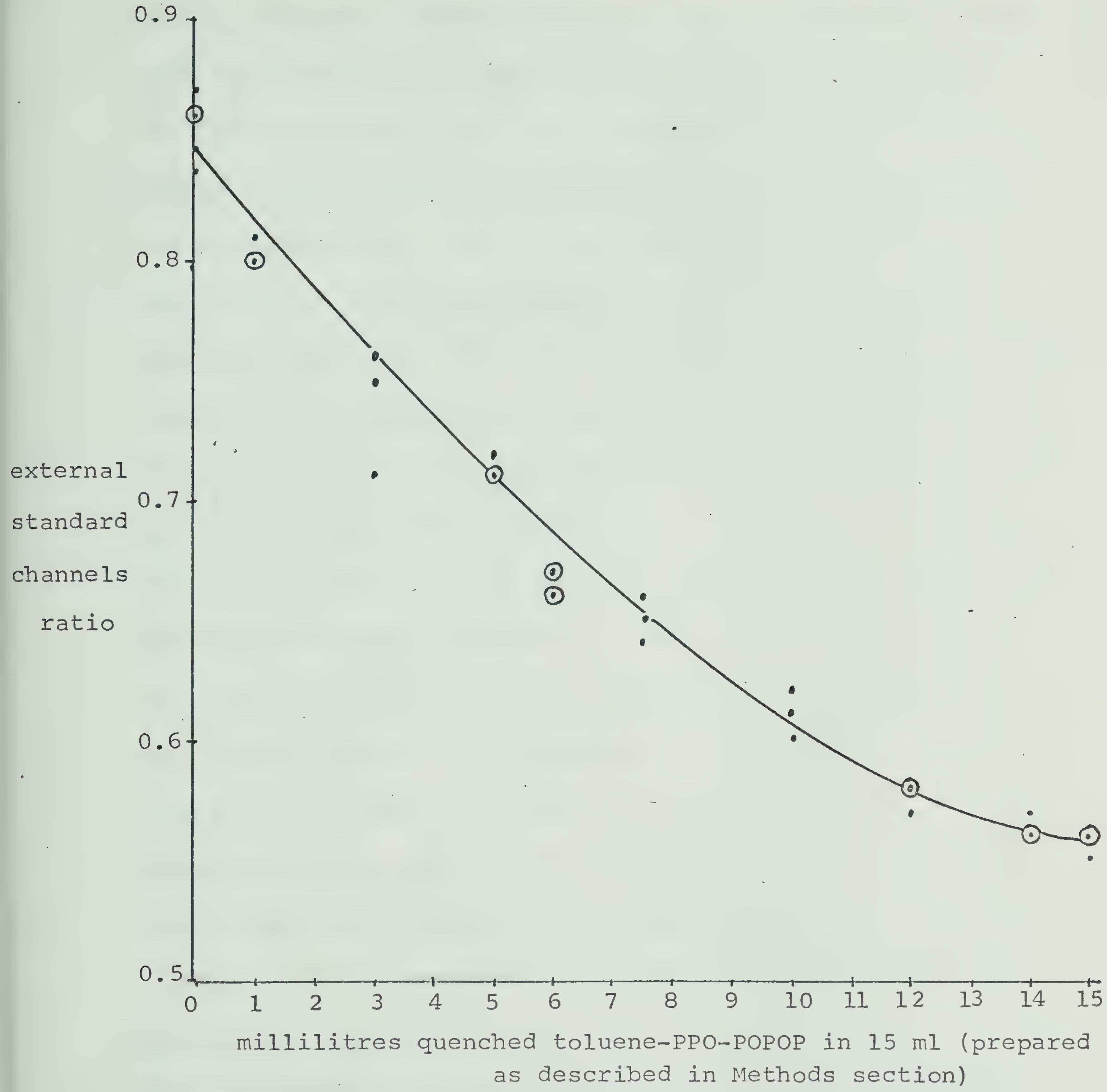


FIG. VI: Relationship between external standard channels ratio and amount of quenching agent added.
Beckman S-200 B Scintillation Counter
time: 20 min.

and the external standard channels ratio. The latter varies while the counting efficiency remains relatively constant. What variation there is in the counting efficiency is not related to the amount of quenching agent added. The average counting efficiency of all of the samples given in Table VI was 97.0% with a standard deviation of 1.7 (coefficient of variation was 1.8%). It was assumed that all DAO assay samples counted on the LS200-B gave 97% counting efficiency and the external standard channels ratio was not used to determine the exact efficiency. This assumption gave efficiency values as reliable as those obtained from the quenching curves used with the Nuclear Chicago Instruments. External standard channels ratios on the LS200-B were not as readily reproduceable (i.e. for the same sample vial) as those obtained with Nuclear Chicago scintillation counters. It appears that the external standard channels ratio on the Beckman instrument is sensitive to something other than materials that quench carbon-14 beta particles. I am informed by the Beckman representative that adjustments can be made to the normally sealed external standard channels to permit measurement of relatively small amounts of quenching and to make the ratios values more reproduceable. Such adjustments cannot be made on an instrument that must serve several workers having different types of

samples. Completely unquenched toluene-PPO-POPOP samples were counted with 97.5% efficiency on the LS200-B. An argon-flushed standard supplied by the makers was counted at 95.2% efficiency.

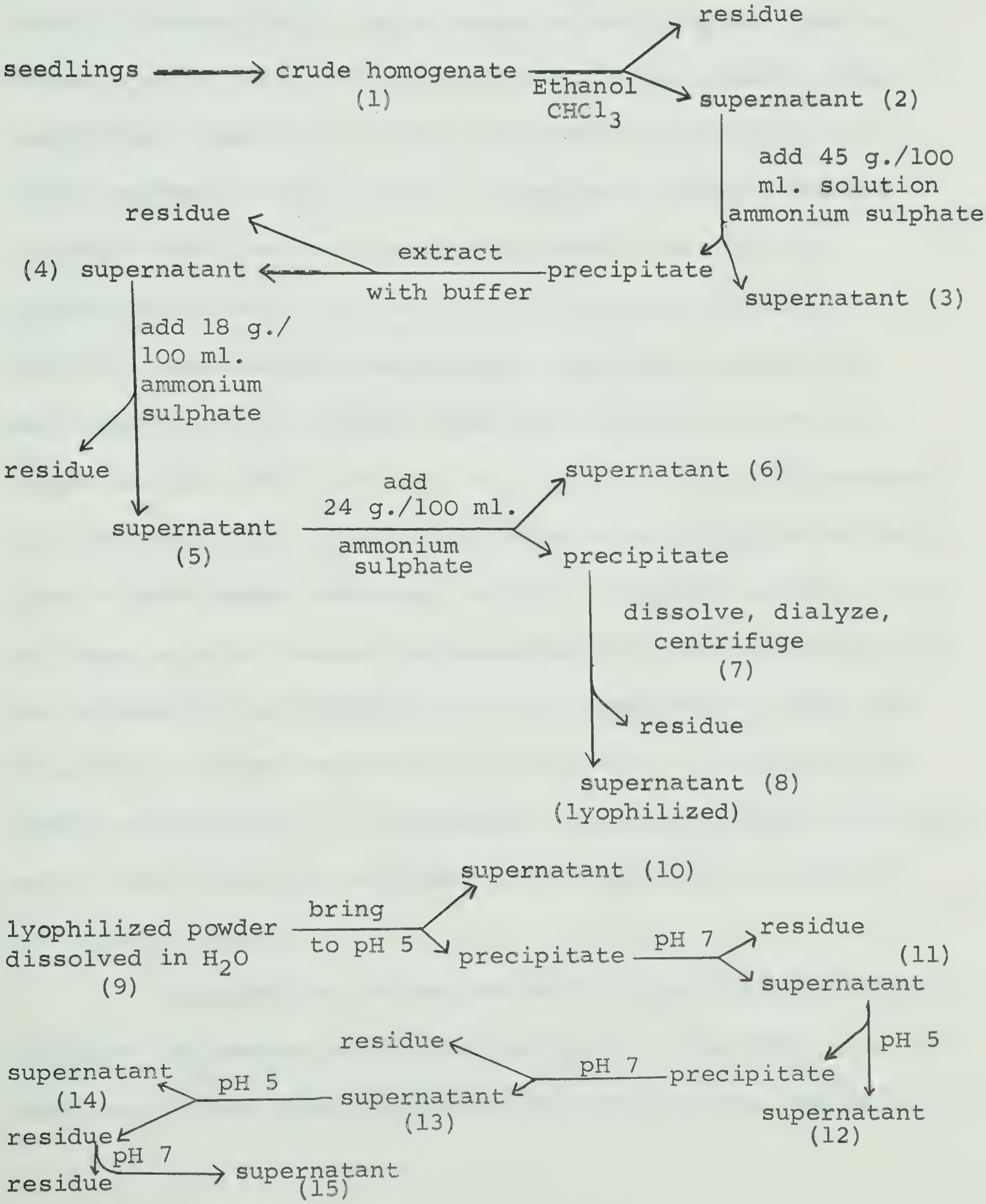
The efficiencies obtained with the LS-200B were almost unbelievably high. Careful checks were performed on the toluene-^{14}C solution to see if it might have lead to high efficiencies by being more active than it was assumed to be. Use of a freshly diluted standard solution and samples of the undiluted standard revealed that the high efficiencies were not the result of some error in the dilution of the standard. Unless the standard itself was faulty, it can be assumed that the high efficiencies were real. The unusual results obtained from the LS-200B were found in three different runs, widely separated by time and so, apparently, such behavior is typical of the instrument.

C. DEFINITION OF THE DIAMINE OXIDASE UNIT

Most workers express diamine oxidase in terms of µl. O_2 consumed in one hour (390). For the purposes of this thesis, one unit (U.) of diamine oxidase is defined as that amount of diamine oxidase that will catalyze the oxidation of 1×10^{-2} µmoles of cadaverine per hour at 30°-34° and at pH 7.2. As oxygen and cadaverine are consumed in equimolar amounts this unit may be calculated as being equivalent to an oxygen consumption of 0.0446 µl. of oxygen.

D. ISOLATION AND PURIFICATION OF DIAMINE OXIDASE FROM LUPINES AND PEAS

A diagrammatic summary of the purification procedure used is given below:



The volume of solution and the specific activity at each stage are given in Table VII. The numbers in Table VII correspond to the numbered steps in the above diagram. The overall purification obtained was only 52.3X, but examination of Table VII shows that a large amount of activity was lost at stages 4 to 5, 11 to 13, and 13 to 15. It is possible that insufficient ammonium sulphate was added to precipitate all of the enzyme and that lupine (as opposed to pea) diamine oxidase either does not precipitate effectively at its isoelectric point or has an isoelectric point below pH 5. Physical losses during purification are also a possibility. Mann reports that a similar procedure purified pea DAO by a factor of 300 (389). Of the total activity initially present, 5.7% was recovered. A previous preparation of lupine DAO had given a much better recovery: 26.8%. It appears possible that spillage or error during the procedure may have contributed to the extremely poor recovery in the run reported in Table VII. From 634 g. lupine seedlings, 17.3 mg. (64 U.) of pure white powder was obtained. In a previous run, 698 g. lupine seedlings gave 11 mg. (89.6 U.) of powder with a specific activity of 8.1 U./mg. protein.

Preparations of pea DAO had a higher specific activity, although the powders were brown in colour. From 890 g. of pea seedlings (grown under illumination) gave 43.7 mg. (694.8 U.) of

step number	volume (ml.)	specific activity (U./mg. protein)	purification
1	720	0.045	-
2	780	0.001	0.022X
3	870	0.000	-
4	390	0.119	2.64X
5	410	0.000	-
6	450	0.000	-
7	55	0.067	1.49X
8	160	-	-
9	40	3.533	-
10	120	0.091	-
11	36	2.065	45.9X
12	64	9.910	-
13	60	2.188	48.6X
14	105	3.730	-
15	80	2.353	52.3X

TABLE VII. Purification of lupine diamine oxidase. 'Purification' was calculated by dividing the specific activity at any stage by the specific activity of the initial homogenate

specific activity 15.9 U./mg. protein. The overall recovery was 48.2% of the activity initially present. DAO was prepared from green pea seedlings and from etiolated pea seedlings. Both were equally good sources, but etiolated seedlings gave less strongly coloured solutions and so were preferred. It was observed that the total number of units of DAO present increased slightly after treatment of the plant extracts with ethanol-chloroform during the preparation of pea seedling DAO. This was not observed when lupine seedling DAO was prepared and may represent the removal of some inhibitory materials.

The preparation method could be improved to give more active lupine DAO, but time and the quantity of lupine seeds available precluded this. Two preparations of lupine DAO were made as well as four preparations of pea seedling DAO.

A light yellow-brown colour was noted in buffer that was used to dialyse the first preparation of lupine DAO. Two litres of the solution were frozen and lyophilized and a concentrated solution of the dry material was made. The absorption spectra of this solution in the visible and U.V. were examined with a Perkin-Elmer Model 202 U.V.-visible spectrophotometer. There was little absorption in the visible, although dilute solutions of pyridoxal phosphate gave distinct absorptions in the visible. In the U.V. strong absorption was found near 200 mμ. This absorption maximum shifted towards

longer wavelengths when the solution was made basic. The U.V. spectrum bore no resemblance to that of pyridoxal phosphate. Phosphate buffer at pH 7.0 was found to give an identical spectrum to that of the unknown and gave similar shifts with changes in pH. It was concluded that the only material present in the solution that absorbed significant amounts of light was phosphate. No evidence for the presence of pyridoxal phosphate was found.

E. ASSAY OF DIAMINE OXIDASE BY HOLMSTEDT AND THAM METHOD

Putrescine was used as a substrate to test the assay for usefulness in determination of pea seedling DAO. The absorbance at 430 mμ after incubation of DAO with putrescine and *o*-aminobenzaldehyde was found to increase in a nearly linear fashion with increasing DAO concentration. Absorption spectra of the coloured products formed with putrescine and cadaverine were prepared using a Perkin-Elmer 202 spectrophotometer. The putrescine product absorbed maximally at 435 mμ while the product from cadaverine had its maximum absorption at 450 mμ. Cadaverine was used as substrate and it was found that the absorbance (read at 450 mμ.) varied linearly with the amount of DAO added. Figure VIb shows this relationship. It was attempted to obtain a value for the molar absorbancy coefficient for the absorption of the coloured product from cadaverine at 450 mμ by permitting the reaction

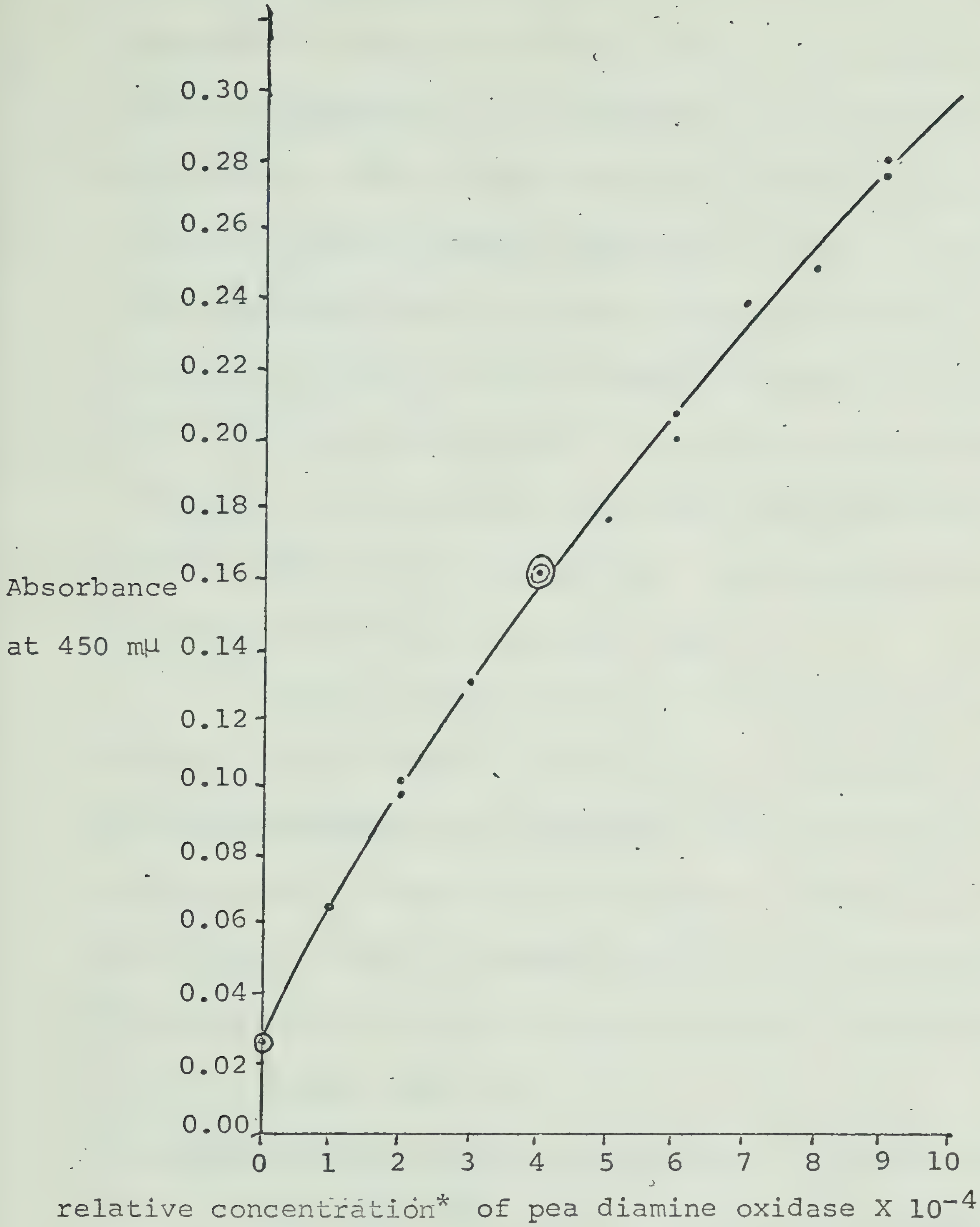


FIG. VIb: Holmstedt and Tham assay for diamine oxidase activity using cadaverine as substrate.
Reaction mixture: 2.5 ml. 0.005 M. o-aminobenzaldehyde in pH 6.8, KPB, 3.5 ml. KPB pH 6.8, 1.0 ml. DAO solution in KPB pH 6.8, 0.5 Ml. 0.1 M cadaverine in KPB pH 6.8.
Incubated 3 hr. followed by addition of 1 ml. 10% trichloroacetic acid.

* A concentrated DAO solution was diluted to give the relative concentrations shown.

to proceed to completion in the presence of known amounts of cadaverine. When this was done, the values obtained were not at all feproduceable. It is possible that some other factor affected the reaction when substrate, rather than enzyme, was limiting. For the results obtained with this method to be useful and comparable to those from other methods, it was necessary to know the exact amount of cadaverine consumed. This could not be determined in the absence of a molar absorbancy coefficient and for this reason the method was not further used. A molar absorbancy coefficient has been obtained for the product from putrescine (425).

It was attempted to use this method to study the activity of DAO towards lysine; however, when DAO, lysine and *o*-aminobenzaldehyde were incubated together no colour development was observed. As DAO has been shown to oxidize L and D lysine (see below) it must be assumed that the Δ'-piperideine-6-COOH formed from the lysine is unable to react with *o*-aminobenzaldehyde to form foloured compounds.

F. DIAMINE OXIDASE ASSAY BY THE METHOD OF OKUYAMA AND KOBAYASHI

The method devised by Okuyama and Kobayashi is based upon the toluene solubility of the reaction products of the DAO catalyzed oxidation of cadaverine or putrescine. At pH 8 to 11 the amount of substrate extracted was not significant (426). Any factor that affects the extraction efficiency

will also affect the ultimate results. Thus the effects of pH of the amount of excess sodium bicarbonate, and of the amount of protein present were checked. When inhibitor or pH runs were made, blanks were used to correct for any changes in the background amounts of unreacted cadaverine extracted.

(1). Effects of pH on the Extraction of Cadaverine

Okuyama and Kobayashi found that between pH 8 and pH 10 no changes in the amounts of cadaverine extracted occurred. At pH values higher than 12 cadaverine began to be extracted (426). They used sodium bicarbonate saturation to produce a constant pH of 8.3 for the extraction. I have found the pH of pooled samples after extraction to be 8.66 at 29°. When phosphate buffers of different pH containing radioactive cadaverine were extracted no significant changes in the amount of extracted radioactivity were noted between pH 2 and pH 12.1. When buffer alone (KPB-BSA) was extracted a background of 34.8 dpm. was obtained. When the same buffer (at pH 7.2) containing 0.100 ml. of radioactive cadaverine (5 mM., 0.0167 mC./mmole) was extracted a background of 81.3 dpm. resulted. This background changed very little with pH. Such an observation bears no surprise as cadaverine exists in solution as a cation at pHs less than 11.

(2). Effect of Quantity of Sodium Bicarbonate on Extraction Efficiency

Approximately 200 mg. sodium bicarbonate were used

by the originators of this method to stop the reaction and to fix the pH (426) of the 2 ml. of reaction mixture. I found that somewhat more sodium bicarbonate was required to ensure saturation. To test the effect of varying amounts of $NaHCO_3$, samples containing diamine oxidase and cadaverine had weighed amounts of $NaHCO_3$ added. The results (averages of two samples) are summarized below:

mg. $NaHCO_3$	dpm. extracted
200*	866.0
300	824.9
400	801.9
600	779.8

(* no visible excess at room temperature)

A three-fold increase in the amount of bicarbonate produces a 10% decrease in the amount of radioactivity extracted. Change in the amount of $NaHCO_3$ from 200 to 300 mg. causes a 4.7% decrease in the extracted radioactivity. The coefficient of variability for similar samples (with constant amounts of $NaHCO_3$) was 4.5% (standard deviation expressed as a percentage of the mean). It may be concluded that it is important that the amount of excess sodium bicarbonate should be kept constant, but that small changes in this amount are of little significance.

The effect of the total volume of fluid used in the extraction procedure was also checked. Samples were extracted with a total of 14 ml. or 12 ml. in place of the

usual 15 ml.

volume ml.	dpm. extracted
15	841.1
14	804.0
12	702.8

A reduction in volume of 1 ml. decreases the extracted radioactivity by 4.4%, a volume decrease of 3 ml. decreases the amount of radioactivity extracted by 16.4%. This shows that small variations in the total volume have little effect, but that variation in the total volume of more than 1 ml. does produce significant decreases in the amount of radioactivity extracted.

(3). Effect of Protein Concentration on Extraction Efficiency

Protein concentrations in actual samples may vary widely. It was therefore important to assess the effect of protein concentration on the extraction efficiency. Samples containing the same amounts of pea DAO and cadaverine (in a total volume of 2.0 ml.) were incubated for 1 hour. The reaction was stopped with aminoguanidine and $NaHCO_3$ and 0.1 ml. of concentrated BSA solutions in KPB was added to produce final protein concentrations from 1.51 mg./ml. to 3.12 mg./ml. The following results were obtained (figures are averages of two samples:

protein concentration mg./ml.	dpm. extracted
0.20	841.1
1.51	806.8
1.92	812.4
2.12	776.1
2.32	792.2
2.62	771.8
3.12	851.4 (average of 4 samples)

The greatest change produced by changing protein concentration was an 8.2% decrease in the amount of radioactivity extracted. A 1% increase was found at the highest protein concentration used. There is no apparent pattern to the changes in extraction efficiency produced by changes in the protein concentration. It appears that increasing protein concentration increases the randomness of the assay rather than decreasing the extraction efficiency. Changing protein concentrations did not affect the values of the blanks. When unusually high protein concentrations were used more replicate samples were required to obtain reliable results. The protein concentration in a typical DAO assay sample was 0.66 mg./ml. Levels higher than 1.5 mg./ml. were not attained.

(4) Reproduceability of the Method

To test the reproduceability of this method, 10 samples, each containing 0.8 ml. BSA-KPB, 0.1 ml. 0.5 mg./ml. catalase in BSA-KPB, 1.0 ml. of pea DAO solution 5 X 10^{-4} mg./ml. (in BSA-KPB), and 0.100 ml. of 0.04 mC./mmole specific

activity 0.5 mM cadaverine in pH 7.0 phosphate buffer 0.01M were incubated and extracted according to the details given in the methods section. The values listed below were obtained (all had identical external standard channels ratios):

cpm. extracted	deviations (cpm.) from the average
640.0	-81.7
745.9	24.2
714.6	- 7.1
717.9	- 3.8
735.7	14.0
748.5	26.8
742.9	21.2
730.9	9.2
701.3	-20.4
739.5	17.8

average = 721.7 cpm. = 841.1 dpm.

The average deviation (absolute) was 3.1%, the standard deviation was 32.5 cpm. and the coefficient of variability was 4.5% (standard deviation as a percentage of the sample average). The "standard error" - the percentage deviation at 90% confidence level (i.e. if a large number of determinations are made the deviation from the mean would be less than or equal to the computed error in 90% of the determinations) for an accumulation of 7,000 counts is 6.5% (cf. table and discussion in reference 649). It is concluded that the results obtained with this method are satisfactorily reproduceable.

(5). Nature of the Reaction Product

Okuyama and Kobayashi were able to demonstrate that the materials extracted resembled piperideine polymers when separated by paper chromatography (426). Despite a total of eleven different attempts, I was unable to repeat their work. Many procedural variations were employed and chromatography on paper and on cellulose thin layers with the solvents described by Okuyama and Kobayashi (426) and Mann and Smithies (331) was used to separate the reaction products. Generally, DAO was allowed to act upon an ample amount of high specific activity cadaverine for several hours. The reaction mixture was then saturated with sodium bicarbonate and extracted several times with toluene (emulsions were broken by centrifugation). The toluene was removed (usually by *in vacuo* evaporation) and the residue taken up in a small amount of water. Counting of paper strips with a Nuclear-Chicago Actigraph III or of areas from cellulose plates by scintillation counting revealed the presence of low levels of radioactivity that was poorly localized.

Staining for the products was done with *o*-aminobenzaldehyde. It was found that a 3% fresh solution in 50% aqueous ethanol was best, although never really satisfactory. The reaction was slow, and it was essential to keep paper or

plate moist with the reagent for about 30 min. for proper colour development.

Although it was not found possible to demonstrate by paper chromatography of the extracted radioactive reaction products that they resembled piperideine polymers, this character of the extracted materials was confirmed when they were shown to develop the colour typical of o-aminobenzaldehyde derivatives of the products of cadaverine oxidation when incubated with the reagent. The absorption spectrum resembled that of o-aminobenzaldehyde derivatives of cadaverine oxidation products.

The stability of the compounds extracted from cadaverine-DAO reaction mixtures was studied by the originators of this method who found that decomposition of the bases before extraction into toluene could contribute, at the most, slight losses in the amount of toluene extractable materials.

(6). Responses to Changes in Enzyme Concentration

The method was reported to give a linear response to increases in enzyme concentration up to a certain level, after which the response to further increments in DAO concentration leveled off (426).

Figure VII shows the response to increases in pea DAO concentration. DAO solutions (1 ml.) containing from 1 µg. to 0.1 µg. of 15.9 U./mg. specific activity pea DAO in

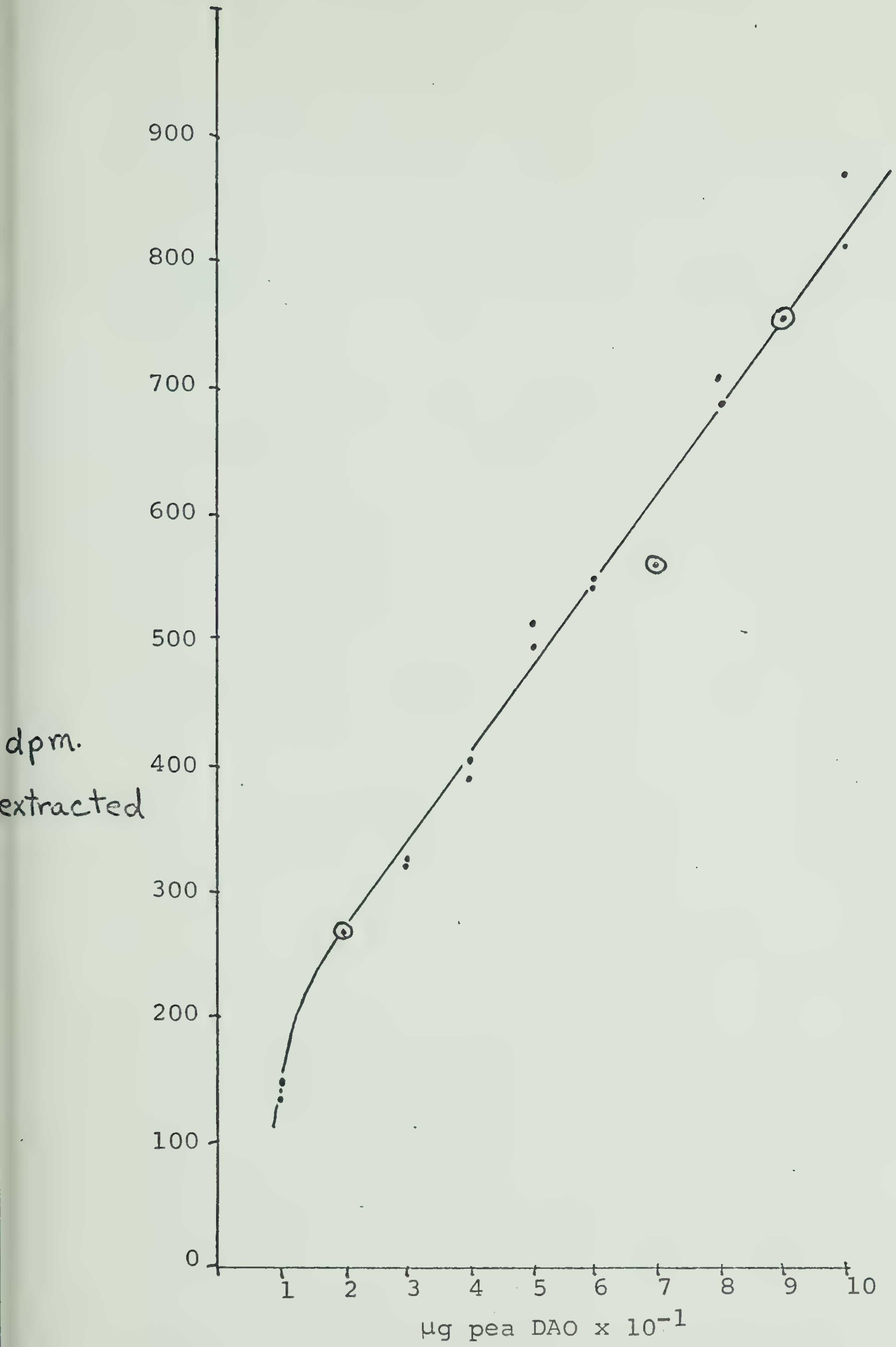


FIG. VII: Assay of pea diamine oxidase by the method of Okuyama and Kobayashi. Reaction mixture: all solutions in pH 7.0 KPB: 1.0 ml. DAO solution, 0.8 ml. buffer, 0.1 ml. 0.5 mg./ml. catalase, 0.100 ml. 0.04 mC./m mole 0.5 mM cadaverine $-^{14}C$., incubated 1 hr. at 33°, samples extracted as described in methods sections. Circles represent duplicate values.

pH 7.0 KPB were incubated with 0.1 ml. of 0.5 mg./ml. catalase in pH 7.0 KPB, 0.8 ml. pH 7.0 KPB and 0.100 ml. 0.04 mC./mmole specific activity 0.5 mM cadaverine-(1,5)-^{14}C in 0.01M phosphate buffer pH 7.0. Over most of the enzyme concentration range the response is linear.

Figure VIII illustrates the response to three levels of lupine diamine oxidase. The reaction conditions were the same as described for Figure VII. The three points (each is an average of 3 samples) on this curve nearly fall in a straight line. Both figures show that the extrapolated line fails to pass through the origin. That is, extrapolation to infinite dilution results in some activity above background. It must be presumed that the curve rises sharply (probably in non-linear fashion) at very low DAO concentrations.

This is illustrated by Figure IX, which shows the radioactivity extracted by the method described in section II from solutions containing varying amounts of pea DAO incubated with 0.100 ml. labeled cadaverine of 0.04 mC./mmole specific activity (0.5 mM) under the standard conditions. Each point on the curve is an average of two samples. It can be seen that activity is low at 1 to 10 nanograms DAO, then rises sharply and gives a linear response to increases in enzyme concentration between 0.1 and 10 μg. DAO. After the 10 μg. level is reached activity falls off or remains constant. This

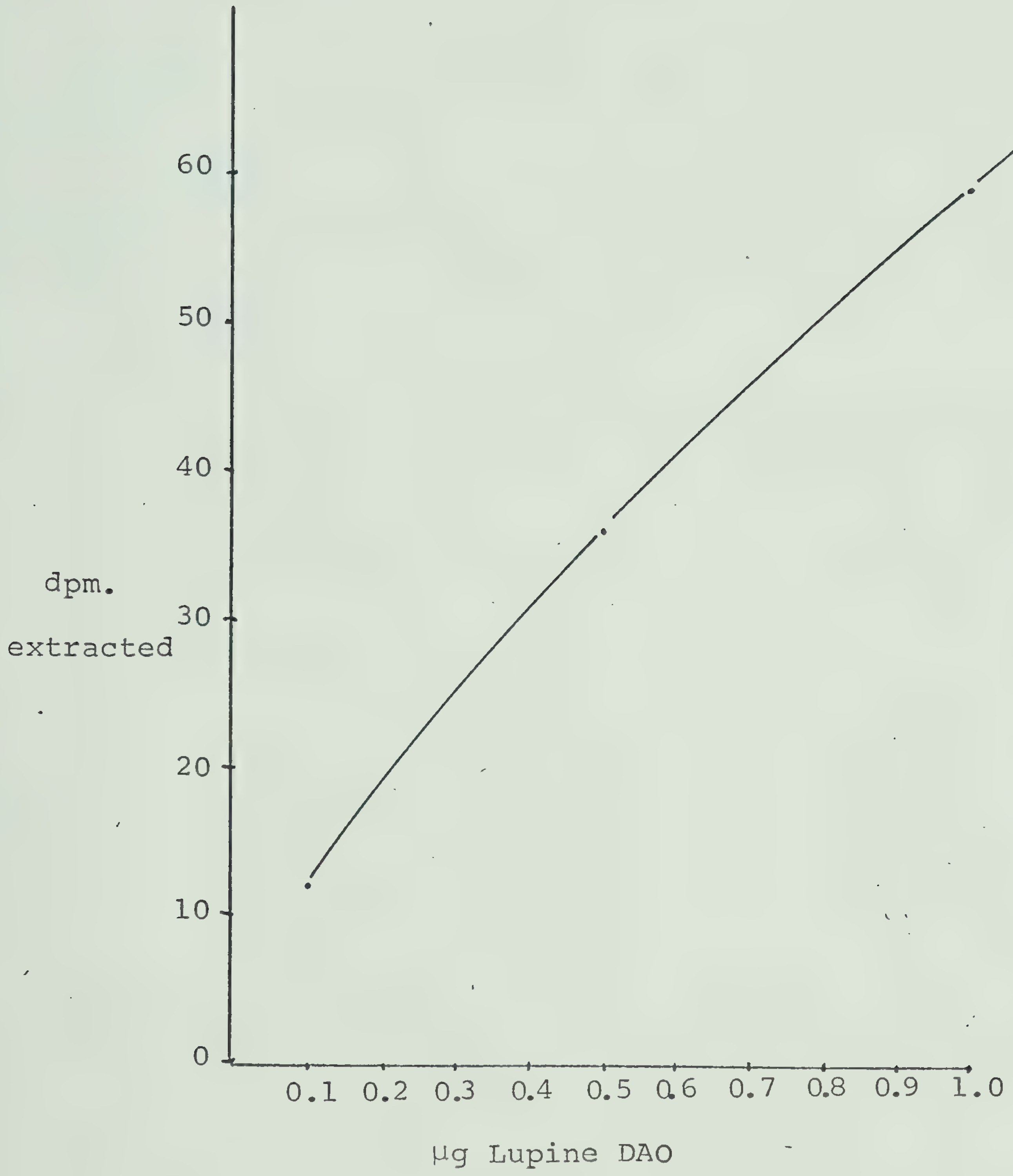


FIG. VIII: Assay of lupine diamine oxidase by the Okuyama and Kobayashi method. Reaction mixture: 1.0 ml. lupine DAO, 0.8 ml. BSA-KPB pH 7.2, 0.1 ml. 0.5 mg./ml. catalase, 0.100 ml. 0.04 mC./m mole 0.5 mM cadaverine -^{14}C, all solutions in KPB pH 7.0, incubated 1 hr. at 33°, samples extracted by procedure outlined in methods section. Points are averages of two values.

behavior is typical of that found in all runs with pea DAO. Lupine DAO was of such low specific activity that such responses were not clearly detectable although their existence is suspected. Other workers consider pea and lupine DAO to be identical (391). If such an assumption is valid (and I have seen no evidence to the contrary) it is likely that lupine DAO does show the same behavior as pea DAO.

It can be seen from Figure IX that the specific activity of the pea DAO increases with dilution. The figures for this are given below and plotted in Figure X.

amount of DAO (mg.) per sample	dpm.	specific activity (U./mg.)
0.1	1,335.9	15.0
0.01	1,362.4	153.0
1×10^{-3}	782.4	881.0
1×10^{-4}	143.6	1617.0
1×10^{-5}	13.7	1540.0
(1×10^{-6})	(4.1)	(4630.0)

The last figure is omitted, as at such low levels, insufficient counting time was allowed to accumulate a statistically significant number of counts. Error in this figure is magnified enormously in the computation of specific activity. Figure X shows that the specific activity is greatly increased by dilution. The behavior of the specific activity at dilutions greater than 10 mg. is uncertain. It appears that it may either drop or level off. There must be a marked decrease in specific activity at very high dilutions - it would be most unreasonable

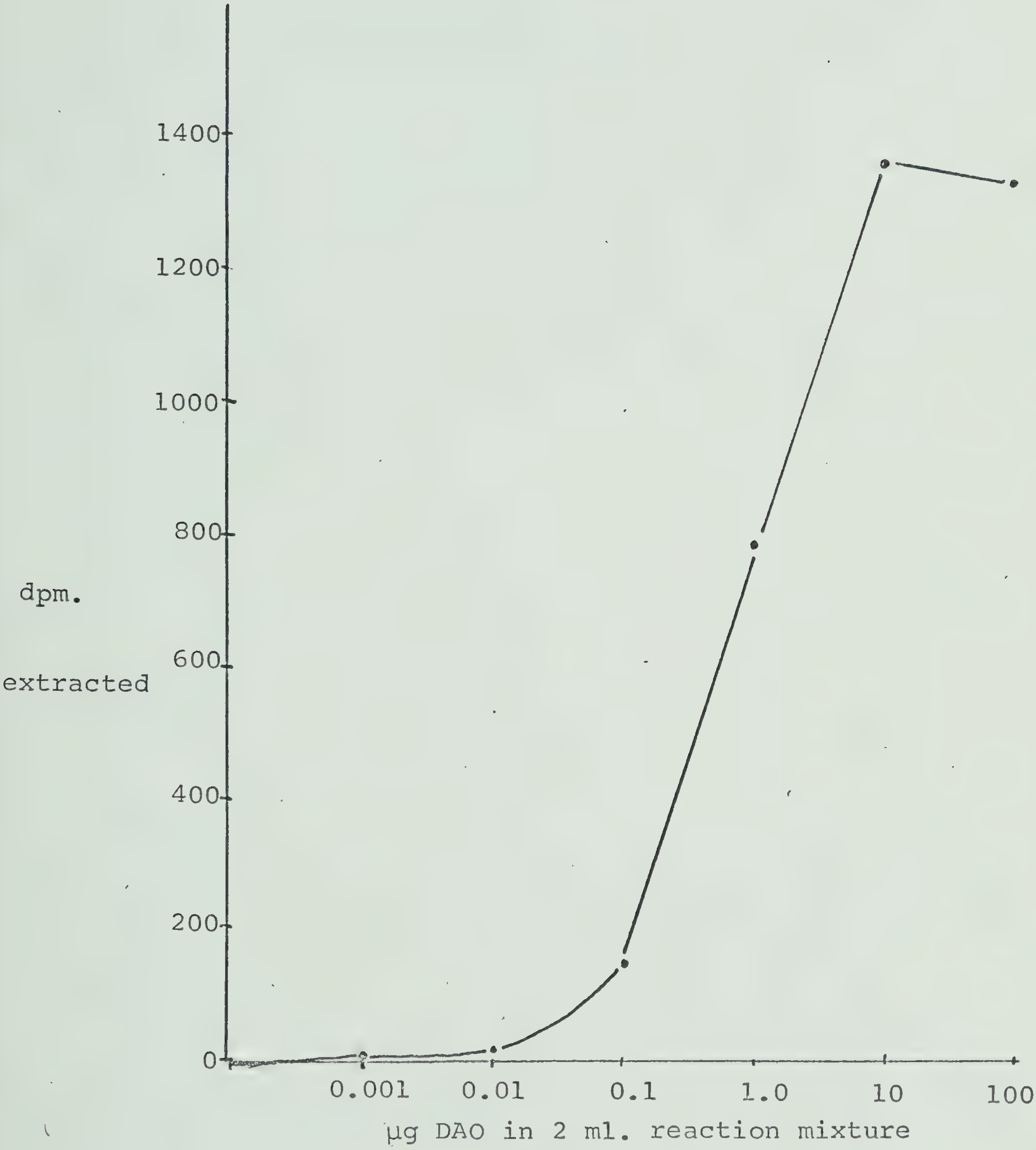


FIG. IX: Change in amount of extractable radioactivity with change in amount of pea diamine oxidase. Reaction mixture: 1.0 ml. pea DAO solution, 0.8 ml. KPB pH 7.0, 0.1 ml. 0.5 mg./ml. catalase, 0.100 ml. 0.04 mC./m mole 0.5 mM cadaverine $-^{14}C$. All solutions in KPB, pH 7.0, incubated 1 hr. at 33°, extraction procedure as in methods section.

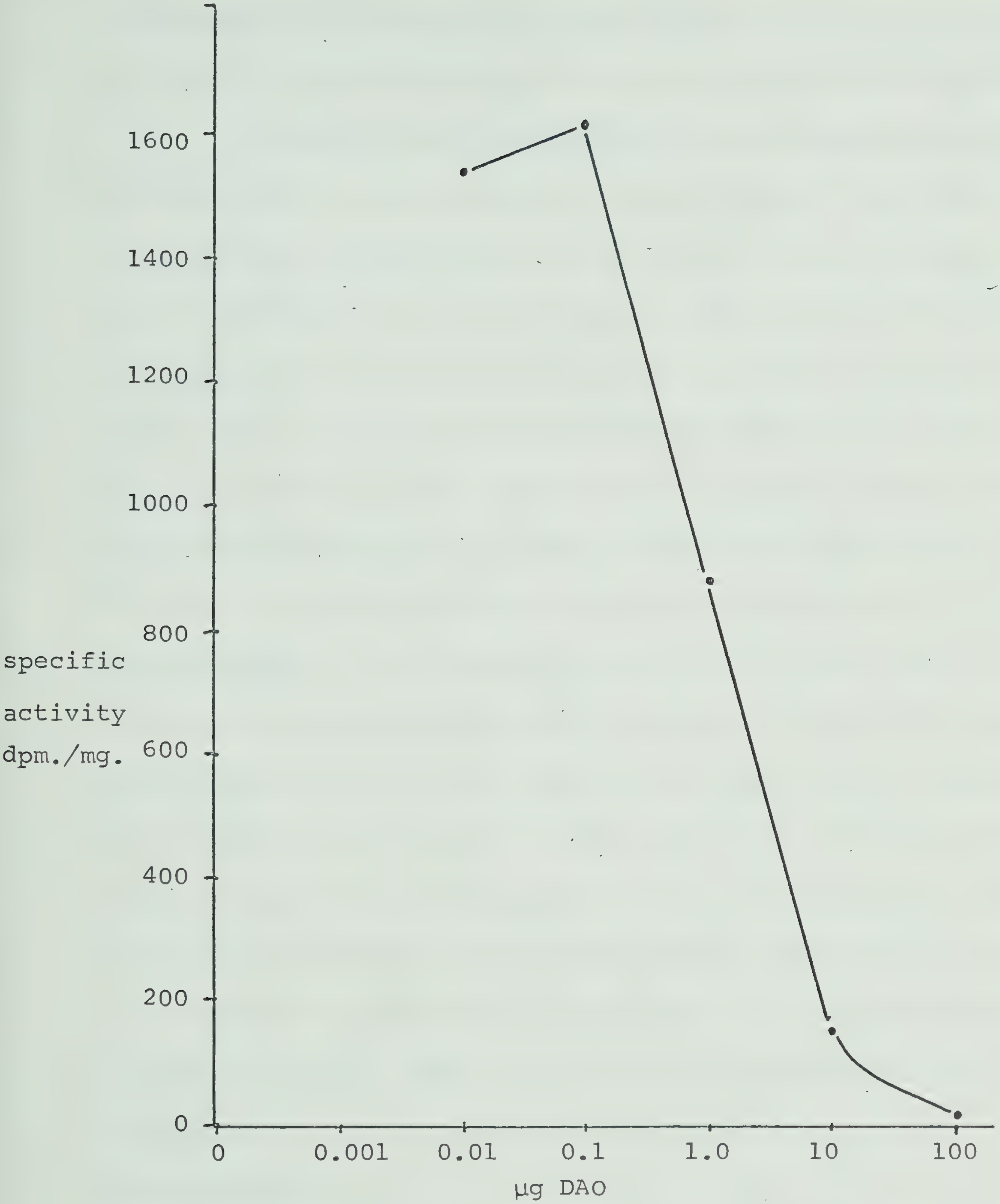


FIG. X: Change in specific activity of pea diamine oxidase with dilution. Conditions the same as those for Fig. IX.

if the specific activity increased continuously to infinite dilution. Such a phenomenon would be of cosmological importance.

Anomalous relations between enzyme concentration and activity are discussed by Dixon and Webb (648). The behavior at very high dilutions could be the result of the presence of some inhibitory material: at higher enzyme concentrations all of this material has been tied up with protein. Traces of metal ions in the water used may cause such behavior (648). Similar curves may also result from the presence of a dissociable activator or coenzyme. At high enzyme concentrations the reaction velocity becomes proportional to the enzyme concentration as the enzyme is saturated with the cofactor. Studies were made with pyridoxal phosphate, cupric ions, and EDTA addition (see below), but none of these had any effect on the non-linear behavior at high dilution. It is possible that; if pea DAO were composed of dissociable subunits (this is possible, although the observed molecular weight is thought to be that of one subunit of animal DAO), as has been suggested by Mondovi et. al. (395), this behavior might result from dissociation into inactive subunits at very low enzyme concentration.

Increase in activity with dilution has been observed with plant extracts as well as with partially purified enzyme preparations. Extracts of bitter lupine gave 376.1 dpm.

extracted when incubated with 0.04 mC./mmole specific activity 0.5 mM cadaverine under standard conditions. When diluted ten-fold an activity of 96.0 was observed. This represents a 155% increase over that which would have been expected if there was a linear relationship between dilution and activity.

This effect makes assay of plant extracts for DAO activity rather difficult. It is important to carry out all extraction procedures under identical conditions to insure that unnecessary and unreproduceable dilutions are not introduced. Assay of plant extracts for DAO activity is probably reliable for the observation of large changes in the amount of activity present: less confidence should be placed in small activity changes detected in plant extracts. The cause of the increase in activity is uncertain: it might be related to the dissociation of some inactivating agent or it might be possible that at very high enzyme concentrations, there is such competition for substrate molecules that fewer diamine molecules are bound exclusively to one protein molecule (*i.e.* many molecules would be bound to two enzyme molecules). The distance between the two ends of a cadaverine molecule (near pH 7) has been reported to be 7.3 Å (615): the overall length of the molecule is 9.1 Å (calculated from data in reference 412). The maximum 'length' of a water molecule is 2.06 Å (data from (142)). It appears just barely possible that two protein

molecules could bind cadaverine if the binding sites were on the surface. It would be of interest to see if the same effect was observed with longer chain and shorter chain diamines. The leveling off of the total amount of extracted radioactivity seen in Figure IX is not the result of substrate limitation - if all the substrate were oxidized, 44,400 dpm. could have been extracted. In conclusion, this method responds in a linear fashion to changes in DAO concentration over a certain concentration range. Below this range the enzyme activity falls in a non-linear fashion and above this range it remains relatively constant.

(7). Efficiency of the Extraction

The data presented above show that a reproduceable amount of radioactivity is extracted from identical samples. It was of interest to know if this represented the total amount of radioactive reaction product. To test this samples containing 1×10^{-3}, 6×10^{-4}, and 1×10^{-4} mg. of pea DAO were incubated with cadaverine under the standard conditions and were extracted as usual with 5 X 5 ml. toluene-PPO-POPOP. Each 5 ml. of extract was added to 10 ml. toluene-PPO-POPOP and was counted. Figure XI shows the amounts extracted from each sample. These results are expressed as percentages of the total amount extracted in five extractions in the following table.

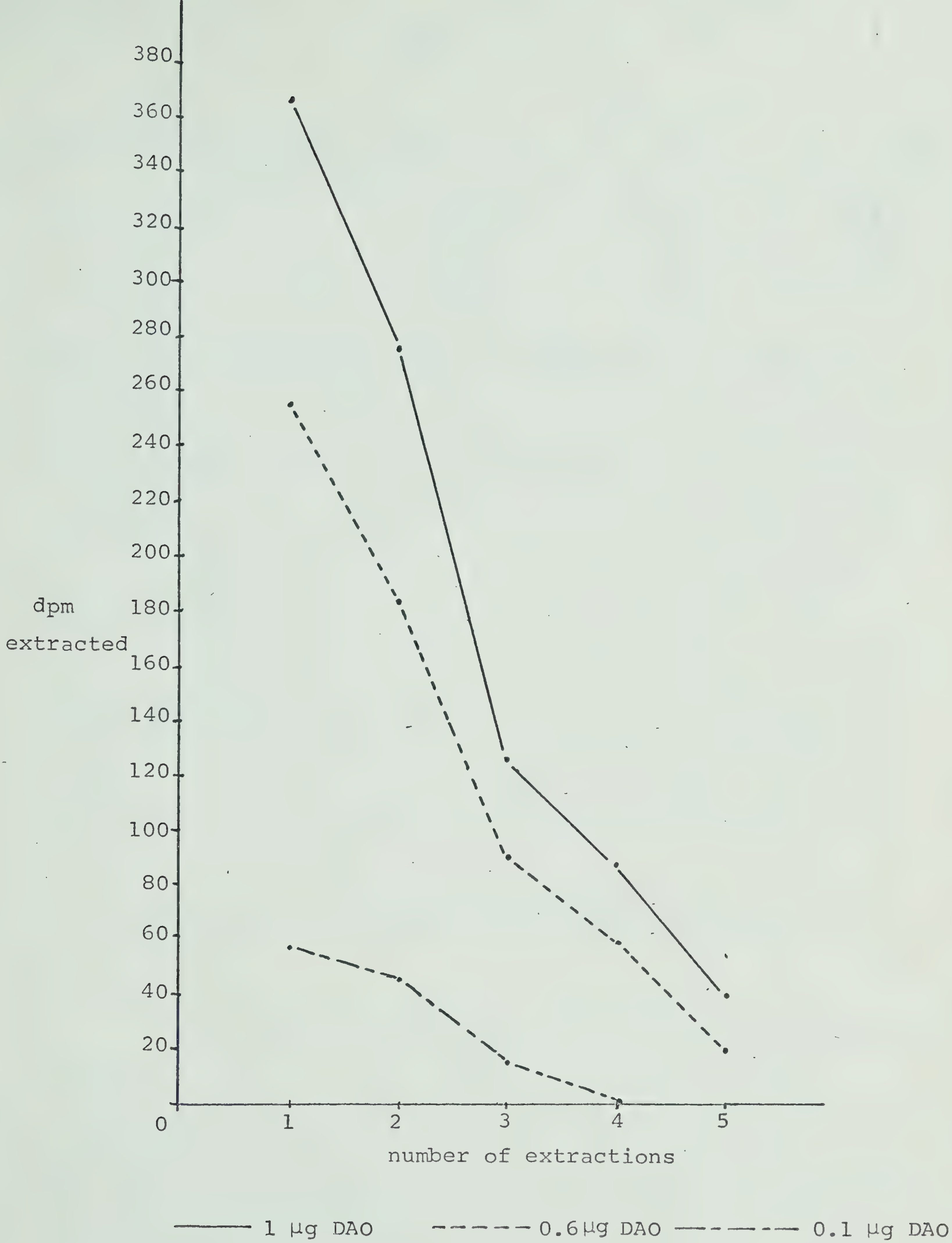


FIG. XI: Efficiency of extraction of reaction product. All points are averages of 2 or 4 values. Reaction mixture: 1.0 ml. DAO in KPB pH 7.0, 0.1 ml. 0.5 mg./ml. catalase in KPB pH 7.0, 0.8 ml. KPB pH 7.0, 0.100 ml. 0.04 mC /m mole 0.5 mM cadaverine, incubated 1 hr. at 33°. Extractions as described in text.

number of extraction	amount of DAO 1×10^{-3} mg.	6×10^{-4} mg.	1×10^{-4} mg.
1	40.8%	41.8%	48.5%
2	30.9%	30.3%	38.6%
3	14.2%	14.9%	13.0%
4	9.8%	9.7%	0 %
5	4.4%	3.3%	0 %

Thus, three extractions suffice to remove 86.5% of the total amount of activity extracted by five extractions. In the case of the lowest DAO concentration examined, three extractions removed all of the radioactivity. Okuyama and Kobayashi found that two or three extractions gave better than 80% recovery. Further extractions became increasingly ineffective (426). Over the linear range of this method variations in the extraction efficiency caused by the presence of differing amounts of product do not contribute significantly to the variability of the results. At high and low concentrations of product (resulting from high or low amounts of DAO) decreases and increases (respectively) in the efficiency of extraction may be significant.

It should be noted that the total activity extracted never represents the total amount of cadaverine oxidized (although it is better than 80% of the total amount oxidized). For this reason, enzyme activities obtained with this technique must be corrected before they can be compared directly with activities expressed in terms of oxygen uptake or ammonia production.

G. SUMMARY

Diamine oxidase from pea and lupine plants was isolated and partially purified. Some of the properties of this enzyme preparation were studied using an assay procedure developed from that reported by Okuyama and Kobayashi. The procedure involved incubation of the enzyme with ^{14}C labelled substrate that was, by the action of the enzyme, converted into toluene-soluble materials that were then counted with a scintillation counter. Although the method gave a linear response to changes in enzyme concentration over a certain range, the activity was found to drop off rapidly at low enzyme concentrations and to level off at high diamine oxidase concentrations. The specific activity of pea diamine oxidase was higher than that of the enzyme prepared from lupines. The specific activity was observed to increase on dilution.

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